THE FATE OF GLUCOSE SUPPLIED AT A RATE SUFFICIENT TO MAINTAIN BASAL METABOLISM

by H. M. DEAN

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

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SUMMARY.

Direct catabolism of glucose administered to a fasting human, or its conversion to fat, does not occur to any large extent within three hours; during this period the body continues to catabolise fat and protein reserves at approximately the same rate as in the fasting state.

The results suggest that in the fasting state there is a depletion of carbohydrate in some part of the body, and that it is this "glucose debt" which is first repaid when glucose is fed.

There seems to be a delay of about 40-60 minutes before equilibrium is reached between glucose supply and glucose disposal by the body.

The rise in metabolic rate observed when glucose is supplied at the rate described is explained as being produced by the heat losses involved in its storage rather than in its catabolism to provide energy directly.

INTRODUCTION.

The body reserves which are catabolised to provide the energy needed by the body under basal conditions are mainly fat and protein which have a higher specific dynamic action than glucose (Lusk, 1931). If glucose could be substituted for the fat and protein catabolised under basal conditions, there should follow an appropriate drop in the observed basal metabolic rate. This hypothesis requires that the feeding of glucose at a rate sufficient to supply basal energy requirements results in virtually complete preferential utilisation of the ingested glucose and that little fat and protein are simultaneously catabolised.

This paper presents the results of experiments conducted to test this hypothesis.

METHODS.

The metabolic rate of healthy male medical students aged from 19 to 22 years was determined both when fasting and when fed glucose, equivalent to their basal needs, for up

to three hours. The level of glucose in their blood was measured at intervals throughout the experimental period.

Measurement of metabolic rate.

The subjects were fasted from 9 p.m. on the previous night and arrived at the laboratory by about 8.30 a.m. On arrival they lay quietly on a comfortable bed for at least 40 minutes before any measurements were made. The room temperature remained constant within 1°C. during each experiment, but varied from 24° C. to 28° C. on different days. Even on the hottest days the subjects remained comfortable and showed no signs of sweating at any stage. The subjects were asked to remain relaxed and to move as little as possible. Any tendency to restlessness was controlled by giving all subjects 16 mg. of phenobarbitone when they arrived in the laboratory. In two experiments the subjects became restless after about 2½ to 3 hours of the somewhat exacting conditions, and were given a further 16 mg. of barbiturate.

Gaseous metabolism was computed from measurements made with a Hartmann-Braun "Basal Metabolic Rate Indicator", in which a gas meter showed the inspiratory volume, and the difference in content of CO_2 and O_2 between the inspired and expired air was measured by a thermal conductivity method. Low resistance glass valves in conjunction with a rubber mouthpiece and nose clip were used to direct the air flow.

Determination of blood glucose.

To obtain multiple samples of blood without repeated venepunctures, a needle with a screw cap containing a rubber diaphragm (Gordh) was inserted under local anaesthesia into an antecubital vein, and remained in place throughout the experiment. It was found to cause little discomfort to the subject during the three-hour period.

Clotting in the needle was prevented by the injection of about 0·1 ml. of heparin (5,000 I.U./ml.) through the rubber diaphragm. To collect a blood sample, the screw cap was removed and sufficient blood was withdrawn to clear the needle of heparin and ensure a true venous sample. After this, the blood sample was collected in a 3 ml. nylon syringe in which the dead space had previously been filled with heparin (1,000 I.U./ml.). After obtaining the sample, the screw cap was replaced and a further 0·1 ml. of heparin was injected through the rubber diaphragm. The samples were kept in a refrigerator and their glucose contents were measured within five hours of collection by the colorimetric method of Folin and Wu (Hawk, *et al.*, 1956) using a Unicam S.P. 600 spectrophotometer. As a test of accuracy, repeated measurements on the one blood sample were found to differ from the mean value by ± 1 mg. glucose/100 ml. of blood.

The dose rate of glucose.

It was proposed to supply glucose at a rate calorically equivalent to the best estimate of fat plus protein catabolism to avoid presenting an excess of glucose. An examination of Drabkin's (1950) distribution of basal oxygen consumption among various organs suggested that glucose equivalent to 70 p.c. of the basal oxygen usage would be within the limits required. This was found to be 1 gm. of glucose per litre of oxygen consumed, as determined by two measurements under "basal conditions" at 30-minute intervals.

The actual intake of glucose ranged from 16-19 gm. per hour for the different subjects, and was supplied as tablets which were consumed at intervals of 5-6 minutes. As a check on any secondary effects of the oral route — e.g., elaboration of digestive juice — the same dose was given by continuous intravenous infusion in two subjects.

RESULTS.

Oral glucose.

In six experiments on six subjects glucose ingestion at the calculated rate began after two preliminary measurements of basal metabolism 30 minutes apart. At intervals of approximately 30 minutes after this, further measurements of metabolic rate and blood glucose were made. This procedure was followed on six subjects for various periods extending from 100 to 200 minutes after glucose ingestion began. Fig. 1 shows the results from all six experiments.

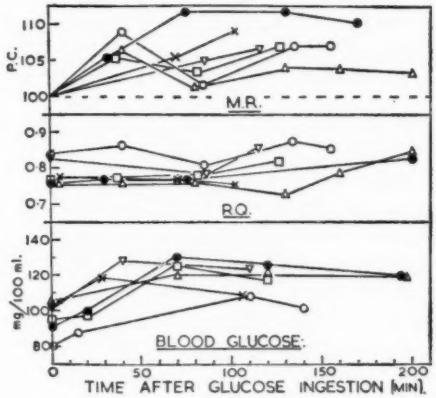

Fig. 1. ***The results of all experiments in which glucose was fed.*** Glucose ingestion begins at zero time and the metabolic rate is expressed as a percentage of the previously determined fasting value.

Metabolic rate (*M.R.*). The two preliminary estimations of M.R. of each individual differed by less than 1·5 p.c., and usually the second value was slightly lower than the first. Within 40 minutes of beginning the administration of glucose the metabolic rate showed an average increase of 7 p.c. above the fasting level, the increase usually being maintained for the remainder of the experiment.

Respiratory quotient (*R.Q.*). The average fasting R.Q. of the subjects studied varied from 0·74 to 0·86 with an average value close to 0·8, which suggested that protein was the dominant catabolite. The two subjects whose R.Q. exceeded 0·8 evidently were oxidising some glucose as well — presumably from their glycogen stores. However, the responses of their metabolic rate and R.Q. to glucose feeding did not differ from those of the other subjects with the lower fasting R.Q.'s.

Steady ingestion of glucose at 70 p.c. of the basal requirement did not cause any increase in the R.Q. within 2-3 hours in spite of the slight rise in metabolic rate. In two subjects observed for a longer period, there was a slight rise in the R.Q. after three hours (Fig. 1).

Blood glucose. This started to rise from the fasting level within about 10 minutes of starting the glucose ingestion, and reached a peak value within 40-60 minutes. In most subjects the concentration fell slightly towards the end of the experiment 2-3 hours after starting the administration of glucose. Whilst the metabolic rate increased by 7 p.c. within 30 minutes, the blood glucose continued to rise after this time without any apparent response in either metabolic rate or R.Q.

To ascertain any effect of the exercise necessarily undergone between the time of awakening and arriving at the laboratory, one experiment was conducted in a similar manner except that the subject slept overnight in the laboratory and the measurements began immediately on waking. The results of this experiment were similar in both direction and magnitude to those obtained previously with the 40-minute preliminary rest.

Three subjects kept to the same schedule except that the glucose intake at the prescribed rate began when they awakened in the morning, and when they arrived at the laboratory they had continued the ingestion for some 3-4 hours. Glucose ingestion was continued during the 40-minute preliminary rest, and during two measurements of metabolic rate and blood sugar. The intake was then stopped and the course of the metabolic rate and blood glucose were followed during the next two hours. The results are shown in Fig. 2.

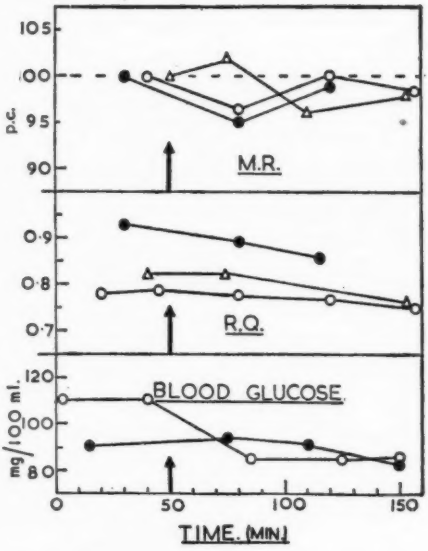

Fig. 2. *The effect on metabolic rate, R.Q., and blood sugar of stopping the ingestion of glucose.* Glucose intake had continued for some 3-4 hours before the first measurements were made, and is represented on the graph as ceasing at 50 minutes (arrow).

M.R. The metabolic rate fell by about 3·5 p.c. within the first 30 minutes—this fall not being statistically significant—and remained at this lower value for the remainder of the experiment.

R.Q. After glucose ingestion was stopped the R.Q. showed a small fall of doubtful significance. In two of the subjects whose R.Q. had remained almost uninfluenced by the glucose feeding at 0·8, there was a small fall in the level to 0·76 within 100 minutes after withdrawal of glucose.

In the other subject the R.Q. had risen to about 0·93 when glucose was fed and appeared to fall at the same rate to 0·86 in 55 minutes.

Blood glucose. The blood sugar as measured while glucose was still being ingested was not as high in these experiments as it was at the end of a previous 2-3-hour period of glucose feeding to the same subjects.

Subject.	*Blood sugar level after 4 hours.*	*Blood sugar level after 2 hours. (previous experiment).*
D.B.S.	91 mg./100 ml.	130 mg./100 ml.
R.M.H.	110 mg./100 ml.	125 mg./100 ml.

After the glucose was withdrawn, the blood sugar fell slowly from this value to a steady level within the experimental period—e.g. in subject D.B.S. the blood sugar fell from 91 mg./100 ml. to 83 mg./100 ml. in 35 minutes, the previous fasting level being 85 mg./100 ml. In subject R.M.H. the blood sugar fell from 110 mg./100 ml. to 85 mg./100 ml. in 35 minutes, the previous fasting level being 95 mg./100 ml.

As a control, the basal metabolism and blood glucose variations were determined on two subjects who were maintained under the same conditions for the same time but who did not consume any sugar.

B.M.R. The basal metabolic rate remained within ± 1 p.c. of the individual's mean, and the blood sugar showed an average variation of ± 1·8 mg./100 ml. These results indicate that when no glucose is fed both the B.M.R. and blood sugar level remain virtually constant.

R.Q. The R.Q. also remained virtually constant.

Subject.	*R.Q. Variation.*
A.J.D.	0·67-0·71
C.D.J.M. ...	0·74-0·76

In a previous experiment these subjects had the following fasting respiratory quotients:

Subject.	*R.Q. Variation.*
A.J.D	0·74-0·77
C.D.J.M. ...	0·76-0·80

Intravenous glucose.

A mechanically-driven syringe was used to infuse a 20 p.c. solution of glucose in saline intravenously at the prescribed rate via a polythene catheter into two subjects. As a transient rise in oxygen consumption followed insertion of the infusion needle, the two preliminary estimates of fasting metabolism were started at least 30 minutes later and while saline alone was being infused at the same rate. The results obtained are shown in Fig. 3 and are essentially similar to those obtained with oral glucose. There was a rise of about 5-6 p.c. in metabolic rate within 40 minutes, and the R.Q. remained substantially unaltered. However, the rise in blood sugar was much more pronounced, going from an average fasting level of 85 mg./100 ml. up to 145 mg./100 ml. within 40-50 minutes.

DISCUSSION.

The ingestion of glucose at a constant rate did not result in a drop in the observed metabolic rate, but rather produced an increase in metabolism of some 6-7 p.c. within 40 minutes of starting the feeding. Because there was no increase in R.Q. during the two-hour feeding of glucose, it appears that the ingested glucose did not replace to any large extent the metabolites being oxidised in this period. The rise in R.Q. after 200 minutes' feeding, shown by two subjects, may be taken as indicating the beginning of direct oxidation of the ingested glucose.

It is most likely therefore that the increase in metabolic rate observed during the feeding of glucose was due to the heat losses involved in its storage and not to its catabolism to provide energy directly. Its conversion to glycogen would not involve any extra output of CO_2 and so would be compatible with an unaltered R.Q. The energy cost of conversion to glycogen – presumably via the hexokinase plus ATP system – would be of the order of 5 p.c. of the glucose converted. Hence, if all the ingested glucose was converted to glycogen, this would account for the greater part of the small rise observed in metabolic rate. This possibility is supported by the experiments of Dann and Chambers (1930) which indicate that the S.D.A. of glucose may, in fact, be due to the energy required for the synthesis of glycogen.

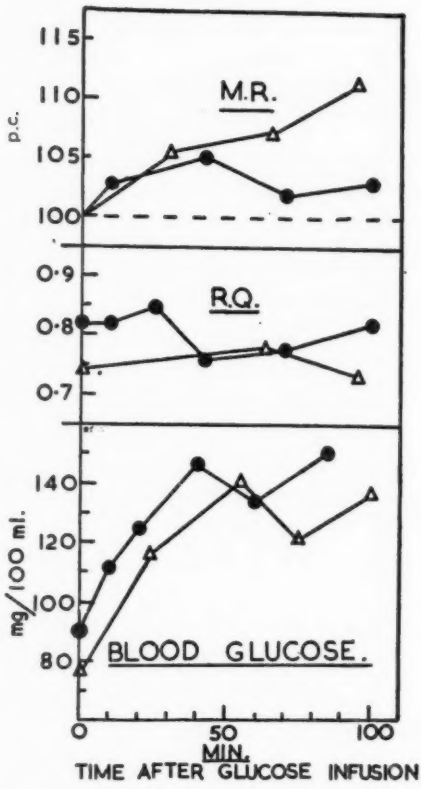

Fig. 3. *The effect of an intravenous infusion of glucose on the metabolic rate, R.Q., and blood glucose in two subjects.* The infusion began at zero minutes, and metabolic rate is expressed as a percentage of the value obtained in the fasting state when saline alone was being infused.

If any glucose were being converted to fat, there would have been a liberation of CO_2 without equivalent utilisation of oxygen and the R.Q. would correspondingly increase. However, no change was observed in the R.Q. and it is therefore considered that the ingested glucose was not stored as fat.

A rise in general tissue level of glucose could account for only a small portion of the total amount ingested. Presumably the concentration of glucose in the blood is in equilibrium at least with that in the extracellular fluid, and so a rise in blood sugar might indicate a rise in tissue glucose, thus accounting for a part of the ingested sugar. For subjects of about 76 Kg. weight, the extracellular fluid volume is of the order of 15 litres, and an increase of 30 mg./100 ml. would therefore represent 4·5 gm. of glucose, i.e. some 25 p.c. of that ingested in the first hour. After this period the blood sugar level fell slightly and consequently no further glucose could be stored in this way.

When glucose was ingested and absorbed at a rate equivalent to 70 p.c. of the basal oxygen usage by young adult males, the blood sugar showed a distinct rise of nearly 30 mg./100 ml. within 40-60 minutes, and thereafter tended to fall slowly. During this period the R.Q. remained very close to the fasting level and only began to rise after about three hours. Apparently, therefore, there was a considerable delay before the rate of utilisation of glucose equalled the rate of its supply. Nevertheless, only 25 p.c. of the glucose given was required to raise the extracellular fluid concentration to the observed level, and the remaining 75 p.c. was removed without any appreciable amount being oxidised. Edwards *et al.* (1934) also did not find an immediate change of the R.Q. after feeding glucose, and concluded that utilisation of the ingested glucose by the tissues takes second place to its use for replenishing glycogen stores.

As ingested glucose would pass through the portal system before reaching the other tissues, two effects might arise:

(a) stimulation of the activity of the stomach and gut;

(b) conversion to other metabolites by the liver.

Both of these might require energy and contribute to the S.D.A. of ingested glucose. The intravenous route, however, presents the bulk of the glucose to other tissues before it reaches the liver, and also avoids any stimulus to activity of the gut. When the glucose was supplied intravenously, only about 50 p.c. of the glucose was removed from the extracellular fluid in a similar time (40-60 min.), indicating that a part at least of the mechanism for glucose disposal lay in the liver. However, in both methods of supplying the glucose, the blood sugar reached its maximum level within 40-60 minutes, thus indicating that it was only by this time that an equilibrium was reached between the glucose supplied and the glucose removed from the blood.

At the time the blood glucose reached its peak, the metabolic rate was increased by about 7 p.c. when the glucose was fed orally and by about 6 p.c. when supplied intravenously, and this difference is probably not significant.

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SOME OBSERVATIONS ON THE PRODUCTION OF BILE BY THE ISOLATED PERFUSED LIVER OF THE RAT

by BEDE MORRIS

(From the Department of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra).

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SUMMARY.

A method for perfusing the isolated liver of the rat in a gas-tight system is described. Under the conditions of the experiments, the livers produced bile and metabolized fat for several hours.

Some observations on the effect of temperature, perfusion pressure and the concentration of fat in the perfusate on the production of bile are reported. It was found that the production of bile was maximal at 38° C. At temperatures of 32 and 42° C., bile flow was significantly reduced. The volume of bile produced was related to the perfusion pressure and increased as the pressure was raised from 5 to 25 cm. of blood. The concentration of fat in the perfusate had no effect on bile production.

There was a significant interaction effect between temperature and perfusion pressure which was thought to be explained by the oxygen requirements of the liver cells in relation to blood flow and bile secretion. The results suggested that bile secretion reached a maximum when all the hepatic sinuses were perfused, provided the oxygen supply to the liver cells was adequate.

INTRODUCTION.

Miller, Bly, Watson and Bale (1951) and Brauer, Pessotti and Pizzolato (1951) have described techniques whereby the liver of the rat can be isolated and perfused under conditions which enable it to perform many of its physiological functions. These workers have used the perfused liver to study the synthesis of protein and the production of bile. When the liver is perfused with a medium containing red cells, bile is produced only as long as the hepatic cells remain viable. The measurement of bile production thus affords evidence of the continued functioning of the liver cells.

In the intact animal, it is difficult to study factors which affect liver function alone without complicating effects occurring elsewhere in other tissues. Changes which alter the haemodynamics of the liver are accompanied by compensatory changes in other regions of the body. The pressure, temperature and rate of blood flow through the isolated perfused liver can be altered readily, however, and thus the effects of changes in these parameters on the metabolism of the

liver can be studied. A series of experiments has been carried out to investigate the transport and metabolism of labelled fatty acids and triglycerides by the perfused liver (Morris, 1960). The apparatus and technique described by Miller *et al.* (1951) have been modified so that the perfusions were carried out in a completely closed system. This enabled all the CO_2 produced by the liver to be collected. The production of bile was measured in each experiment as an indication of the state of the perfused liver. The apparatus used and the method of perfusion are described here, together with the measurements of bile production.

Materials and Methods.

Perfusion apparatus.

The apparatus used was modified from that described by Miller *et al.* (1951) and the details are shown in Fig. 1. All the parts of the apparatus were made of glass or perspex, and all the connections were of "Portex" plastic tubing.

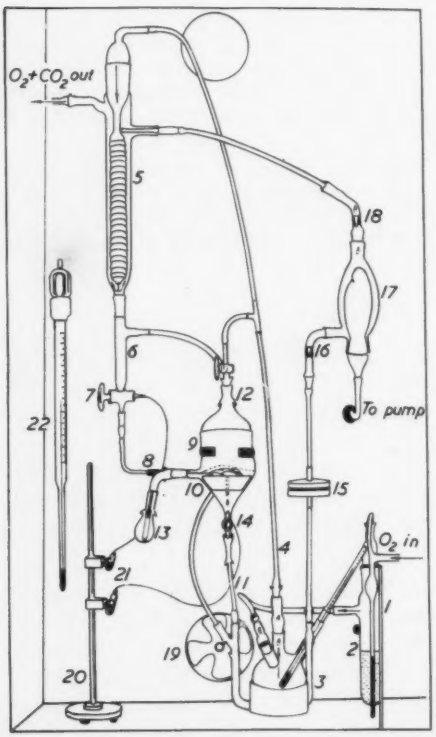

Fig. 1. *Diagram showing the arrangement of the perfusion apparatus.*

1. Mercury manometer calibrated to measure the rate of entry of oxygen into the system.
2. Scrubbing Tower filled with 0·9 p.c. NaCl solution.
3. Perfusate reservoir. Inlet and outlet tubes are situated at the bottom of the flask. Openings at the top of the flask provide for the circulation of oxygen through the flask, the sampling of blood and the recording of temperature.
4. Plastic tube carrying gas from the reservoir to the oxygenation chamber.
5. Oxygenation chamber.
6. Reservoir with the overflow by-pass which returns surplus blood to perfusate reservoir.
7. Two-way tap for obtaining samples of perfusate.
8. Tapered B10 perspex cone with hypodermic needle.
9. Organ chamber.
10. Perspex cone supporting the liver.
11. Plastic tubing which returns the hepatic vein outflow to the reservoir.
12. Top of organ chamber with ground glass joint and tap.
13. Flask holding tube into which bile is collected.
14. Two-way tap for sampling perfusate as it leaves the liver.
15. Perspex filter.
16. Ground glass inlet valve.
17. Pump chamber with finger-stall.
18. Ground glass outlet valve.
19. Fan circulating warm air.
20, 21. Stand and sampling syringes.
22. Mercury contact thermostat.

The Organ Chamber (Fig. 2) was designed to prevent the loss of gases from the surface of the liver. The chamber was 7 cm. internal diameter and 10 cm. in length, and was made with a ground glass joint at the top into which the lid fitted. Two B10 sockets were placed close together on one side of the chamber. A tapered perspex cone, through which a stainless steel 16-gauge hypodermic needle passed, was fitted into one of these sockets. The perspex cone was tapped to receive a rubber grummett over which an aluminium screw could be tightened to make an airtight seal around the needle. The second B10 socket received the flask in which a tube was placed to collect the bile. The lower part of the organ chamber was fitted with a two-way tap, which could be adjusted to allow blood to be sampled as it came out of the liver. The lid of the chamber was joined to a B10 socket into which a tap fitted and allowed the gas within the chamber to equilibrate with the gas in the rest of the apparatus. The liver was supported within the organ chamber by a tapered perspex cone, 6·5 cm. in diameter and 2 cm. deep. The upper surface of the cone was hollowed out to a depth of 2 mm. and a hole 4 mm. in diameter was drilled through the centre. The upper surface of the cone was about 2 mm. below the level of the hypodermic needle which entered the chamber through one of the side arms. A perspex rod could be screwed into the top of the cone and this enabled the cone to be placed in position in the chamber quickly without disturbing the liver. When the liver was in position in the chamber, the rod was unscrewed from the cone and the chamber closed.

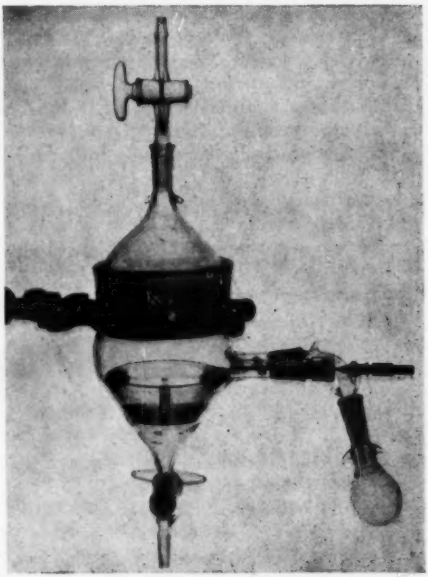

Fig. 2. *The organ chamber used for perfusing the isolated rat's liver.* The photograph shows the perspex cone on which the liver is supported, the flask used for the collection of bile and the tapered perspex adaptor and hypodermic needle to which the portal vein catheter is attached.

The blood reservoir was a 150 ml. flask with four B14 sockets attached. A thermometer fitted through one socket to record the temperature of the perfusate. Samples of blood were taken from the reservoir through one of the sockets and gas entered and left the reservoir through the other two. A tube led into the base of the flask and returned the perfusate to the reservoir. Another tube on the opposite side carried blood from the reservoir to the pump.

Blood was circulated through the system by means of a pump driven by a variable-speed motor. A rubber finger-stall was tied over a grooved B19 cone which fitted inside a glass bulb. This bulb was fitted with inlet and outlet tubes containing ground glass valves which prevented any back-flow of blood. The variable-speed motor drove the plunger of a 20 ml. all-glass syringe which in turn activated the finger-stall and the glass valves.

The blood was oxygenated in a chamber which resembled a condenser. The blood entered the chamber and flowed down the corrugated centre piece of the oxygenator and the oxygen and carbon dioxide exchange took place as the inflowing gas passed back up and out of the chamber.

A reservoir which held approximately 15 ml. of blood was attached to the base of the oxygenator by a B19 cone and socket joint. This reservoir had an overflow arm which could be adjusted to a predetermined height above the portal vein of the liver to give the perfusion pressure required. A two-way tap was situated at the bottom of the reservoir to enable samples to be taken just before the blood entered the liver. The sampling taps were connected to syringes by plastic tubing. A filter constructed of perspex was placed in the system between the main reservoir and the pump bulb. This filter contained a pad of fine nylon gauze through which the blood was drawn and strained free of any small clots.

All the apparatus was assembled within an insulated cabinet, the inner surfaces of which were coated with baked enamel to aid in cleaning. The pieces of the apparatus were secured in the cabinet by clips attached to rods which were freely moveable in a horizontal or vertical plane. This enabled any adjustments in the position of pieces of the apparatus to be made quickly. The box was heated by boost and regulated heating elements. A standard "Sunvic" thermostat controlled the boost element which operated to within 2° C. of the final temperature. The regulated heating element was controlled by a mercury contact thermostat and when the perspex front of the box was in position, the temperature within the box varied less than ± 0·5° C. The heating elements were located in a duct at the rear of the cabinet. The duct opened into the top and the bottom of the cabinet and there was a fan situated at the opening of each duct. These fans circulated hot air through the cabinet after it had passed over the heating elements.

Oxygen was introduced into the apparatus after it had passed through two scrubbing towers, one containing NaOH solution and the other containing 0·9 p.c. NaCl solution. A calibrated mercury manometer acted as a flowmeter, and the flow of oxygen was regulated to about 500 ml./min. The oxygen passed from the tower which contained the saline solution into the main reservoir and from there down through the centre piece of the oxygenating chamber, back up the chamber and out through a side arm leading to scrubbing towers which contained initially carbonate free N/1 NaOH solution. These towers were arranged in pairs and connected by a two-way tap. The gas leaving the apparatus could be directed through either tower by this tap. The gas was then drawn through a tower which contained saturated $Ba(OH)_2$ solution to check the completeness of absorption of CO_2.

Perfusion fluid.

The fluid used for the perfusions consisted of a mixture of 50 ml. of rat's blood, 25 ml. of an electrolyte solution, 25 ml. of 8 p.c. bovine albumin solution, 5 ml. of sodium and potassium phosphate buffer and 5 ml. of amino acid mixture. The blood was obtained from rats of about 330 gm. body weight fed on a stock diet. The rats were anaesthetized with ether and the abdominal aorta was cannulated. The blood was collected onto dry powdered heparin (Boots); 30 mg. of heparin was used for each 50 ml. of blood. The blood was strained through several thicknesses of gauze to remove any clots which may have formed during its collection. Bovine albumin (Armour) was made up as an 8 p.c. solution (W/V) in distilled water.

The electrolyte solution had the following composition: 0·8 p.c. NaCl, 0·04 p.c. KCl, 0·02 p.c. $MgSO_4$, 0·02 p.c. $CaCl_2$ and 0·075 p.c. $NaHCO_3$.

The amino acid mixture was made up so that 5 ml. added to the perfusion fluid provided a content of amino acids similar to that normally found in rat plasma (cf. Henderson, Schurr and Elvehjem, 1948). The mixture contained the following amino acids in 100 ml. of water: 26·6 mg. leucine, 13·7 mg. phenylalanine, 16·8 mg. tryptophane, 26·7 mg. valine, 9·7 mg. histidine, 58·0 mg. lysine, 13·2 mg. isoleucine, 22·7 mg. glycine, 43·0 mg. proline, 22·3 mg. tyrosine, 9·5 mg. methionine, 44·0 mg. threonine, 32·1 mg. arginine.

The phosphate buffer was 4 ml. of 0·1 M Na_2HPO_4 and 1 ml. of 0·1 M KH_2PO_4 (Brauer *et al.*, 1951). The haematocrit value of the final perfusion fluid varied between

19 and 25 p.c. In some experiments fatty chyle obtained from rats was added to the perfusate (cf. Morris and French, 1958).

Operative technique.

Two groups of rats weighing 220 ± 10 gm. and 320 ± 5 gm. were used in these experiments. The starved animals were kept without food for 24 hours before the experiment, but were allowed free access to water. The fed animals were allowed to eat their stock diet of rat nuts up to the time of the experiment.

The rats were anaesthetized with ether and the abdomen opened along the midline. The bile duct was isolated and a plastic catheter (Intramedic PE 10, Adams) tied in place. The pyloric vein, the splenic vein and the cranial mesenteric vein were ligated and the portal vein cleaned for about 1 cm. along its length. A linen thread was passed around the vein near the pyloric vein and left loose. Another tie was passed around the portal vein about 2 cm. from the hilus of the liver and the vein tied off. In most experiments, 0·1 ml. heparin (Pularin, Evans Medical Supplies) was injected through the femoral vein just before the portal vein was tied off. The vein was then cut and a Portex catheter (2 mm. external diameter) inserted and tied in place. The thorax was opened and the sternum cut through on both sides. A plastic catheter (3·0 mm. external diameter) was inserted into the inferior vena cava above the diaphragm, passed caudally until its tip lay near the entrance of the hepatic veins and tied in place. The mediastinal tissues were then cut through and the diaphragm with the liver attached was dissected from the carcass. No attempt was made to ligate the hepatic artery or the inferior vena cava caudal to the liver. The liver, with the diaphragm attached, was placed on the perspex cone, the vena caval catheter passing through the central hole of the cone. The portal vein catheter was cut off to a length of about 2 cm., and the perspex cone and the liver were transferred to the organ chamber. The portal catheter was then pushed onto the hypodermic needle and the perfusion fluid allowed to circulate through the liver. Air embolism did not present a problem as blood flowed back into the catheter when the portal vein was cannulated. As this catheter was cut off just before it was connected to the hypodermic needle, no air bubbles entered the liver.

The liver rapidly regained its normal colour and bile began to flow from the bile duct catheter within 1-2 minutes of establishing the circulation. When the blood flow was established, a gauze pad, moistened in warm saline, was placed over the liver and the lid placed on the organ chamber. All the ground glass joints were checked for leaks and gas was then introduced into the system.

RESULTS.

Blood flow through the perfused liver.

When livers were perfused at a temperature of 38° C. and a pressure of 15 cm., the rate of blood flow rose to a maximum after about the first hour of the perfusion and then remained relatively constant throughout the next three hours. At low perfusion pressures (5 cm. of blood) the rate of flow reached a maximum after the first ½-1 hour and then fell off during the subsequent three hours of the perfusion. Variations in temperature over the range of 32-42° C. had little effect on the rate of blood flow through the liver. The mean rates of blood flow at 42° C. were slightly higher than at 32° C., but the difference was not significant in the series of experiments. The pressure-flow curves for livers perfused at different temperatures are shown in Fig. 3.

The production of bile by the isolated perfused liver.

The rate of bile production was maximal during the first hour after the start of the perfusion; thereafter the rate of production fell to a steady figure and the amount produced in each half-hour up to four hours remained fairly constant.

There was no significant difference in the amount of bile produced by livers from starved or fed rats (Table 1). The weights of the livers from starved rats were, however, significantly less than those from fed rats and when bile production was calculated in terms of flow/10 gm. liver, the livers from starved rats produced significantly more bile (Table 1).

Livers from male rats produced slightly more bile than those from female rats, but this difference was not significant when the results were compared for the same liver weight (Table 1).

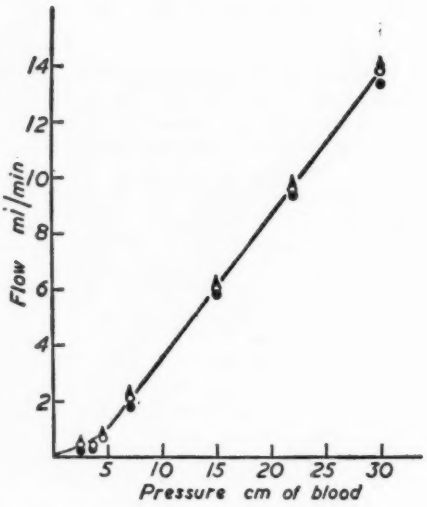

Fig. 3. *The blood flow, perfusion pressure relationships in livers perfused at different temperatures.*

● Perfusions at 34° C.
○ Perfusions at 38° C.
▲ Perfusions at 42° C.

TABLE 1.

The production of bile by the isolated perfused liver of the rat. The livers were obtained from rats weighing 220±10 *gm. and were perfused at a temperature of* 38° *C and a pressure of* 15 *cm. of blood. Mean results are shown together with their standard errors.*

	Starved	Fed	Male	Female
Liver weight gm.	5·48±0·15	6·98±0·34	6·73±0·37	5·73±0·25
Bile production ml./4 hr./liver	1·95±0·10	1·85±0·17	2·19±0·06	1·78±0·06
Bile production ml./4 hr./10 gm. liver	3·56±0·12	2·65±0·11	3·25±0·16	3·11±0·10

The amount of bile produced by the perfused liver was significantly correlated with the weight of the liver. The mean volumes of bile produced in four hours by livers from rats of 220 and 320 gm. body weight perfused at a pressure of 15 cm. and a temperature of 38° C., were 2·95 and 2·81 ml./10 gm. liver respectively. These values were not significantly different.

The effect of changes in perfusion pressure, temperature and fat content of the perfusate on bile production.

There was a progressive increase in the amount of bile produced as the perfusion pressure was raised from 5 cm. to 25 cm. of blood. The most bile was produced by livers which were perfused at the highest pressures and at a temperature of 38° C. Livers perfused at a pressure of 25 cm. of blood produced about six times as much bile as those perfused at a pressure of 5 cm. (Fig. 4). Bile production was well maintained at the higher perfusion pressures and in several experiments, in which livers from rats weighing 320 gm. were used, it reached 1·2 ml. in the first hour. The average hourly production of bile, during these four-hour perfusion experiments, was about 1·0 ml./10 gm. liver. In those livers perfused at a pressure of 5 cm., bile production fell off rapidly after the first two hours and the average bile production was about 0·19 ml./10 gm. liver/hr. (Fig. 4).

Changes in temperature also influenced the amount of bile produced. Over the range of temperatures studied, bile flow was maximal at 38° C. and fell off at temperatures above and below this optimum. Bile flow was less in livers perfused at 42° C. than in those perfused at 34° C. (Fig. 5).

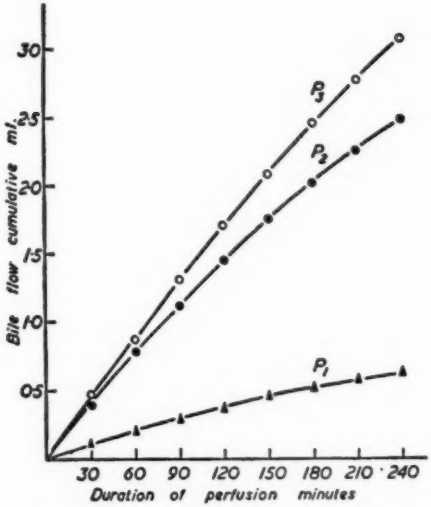

Fig. 4. *The effect of perfusion pressure on the production of bile by the isolated rat's liver.* The cumulative production of bile is shown throughout the 4 hr. perfusion period. Perfusion temperature 38° C.

▲ $P_1 = 5$ cm. blood.
● $P_2 = 15$ cm. blood.
○ $P_3 = 25$ cm. blood.

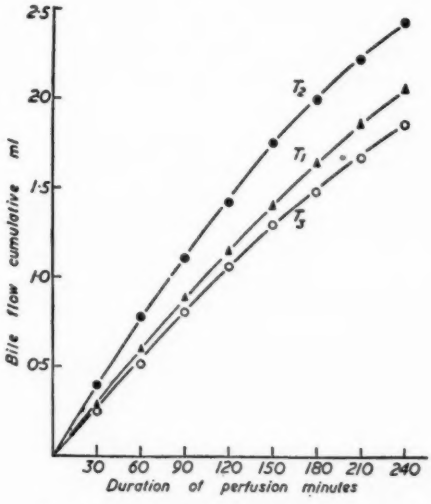

Fig. 5. *The effect of temperature on the production of bile by the isolated rat's liver.* The cumulative production of bile is shown throughout the 4 hr. perfusion period. Perfusion pressure 15 cm.

▲ $T_1 = 34°$ C.
● $T_2 = 38°$ C.
○ $T_3 = 42°$ C.

Variations in the concentration of total fatty acids in the perfusate from 0·25 mg./ml. to 4 mg./ml. had no effect on the production of bile by the liver (cf. Table 2).

The analysis of variance of the effect of temperature, pressure and fat concentration on the production of bile is given in Table 2.

The quadratic components of both the temperature and pressure response lines were significant and there was also a significant linear by linear interaction effect between temperature and pressure. Fig. 6 shows the combined effects of temperature and pressure on bile production. The response to changes in pressure in terms of bile produced in four hours is shown along the interrupted vertical lines. The response to changes in temperature is shown along the solid curved

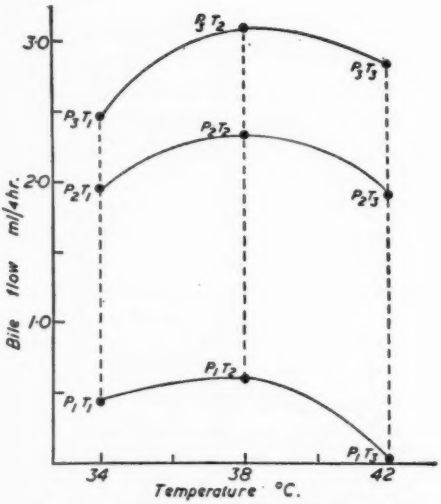

Fig. 6. *Graph showing the effects of temperature and perfusion pressure on the production of bile by the perfused liver.* The interrupted lines represent the responses (ml. of bile produced/4 hr.) to changes in pressure and the solid lines the responses to changes in temperature.

TABLE 2.

Summary of analysis of variance of bile production.

Factor	Levels tested
Pressure (P)	5 cm., 15 cm., 25 cm.
Temperature (T)	34° C, 38° C, 42° C.
Concentration of fat (C)	0·25 mg/ml., 1·0 mg/ml., 4·0 mg/ml.

Source of variation			Df.	Mean square	Significance level
Main effects	P	Linear	1	20·909	***
		Quadratic	1	1·567	***
	T	Linear	1	0·201	—
		Quadratic	1	0·934	**
	C	Linear	1	0·027	—
		Quadratic	1	0·053	—
First order interactions	Linear x Linear		1	0·701	*
	Remainder		11	0·107	
	Residual		8	0·120	

*** Significance P 0·001
** P 0·01—0·001.
* P 0·05—0·01.
— Not significant.

lines. The description of each calculated observation in this factorial experiment is given in the figure.

The production of bile falls off along the P_1 response line to almost zero at 42° C. At perfusion pressures of 15 and 25 cm., however, the rate of decline in in bile production is much less. The amount of bile produced is greater, in fact, at 42° C. than at 34° C. when the perfusion pressure is 25 cm.

DISCUSSION.

The technique of liver perfusion.

The perfusion fluid used in these experiments consisted of whole blood diluted with solutions containing electrolytes, protein and amino acids. The principal difference between this fluid and rat's blood was its reduced red cell content. As the perfusate was equilibrated against oxygen during the experiments, a sodium and potassium phosphate buffer was used to maintain the pH at 7·4 (Brauer *et al.*, 1951).

Other workers have emphasized the importance of reducing to a minimum the period of anoxia which occurs when the liver is transferred from the animal to the perfusion apparatus. In a series of 56 perfusions, the mean time taken to insert the catheters in the portal vein and the inferior vena cava, to dissect the liver from the rat and to establish the perfusion was 5·65 ± S.E. 0·13 minutes. For part of this period the liver received some blood supply through the hepatic artery. When the period of anoxia was about 10 min. or longer, the period of survival of the livers was shortened appreciably.

The inferior vena cava below the liver was not tied off before the liver was dissected from the body. The cut end of this vessel was left open and this provided a useful indication of any resistance to the outflow of blood from the liver. No blood flowed back through the open end of the vena cava provided the liver was arranged correctly in the organ chamber and there was no kinking of the outflow catheter.

The volume of bile produced by the liver during the first four hours was of the order of that produced by the liver of the intact rat. Brauer *et al.* (1951) reported some figures for bile production by the isolated perfused rat liver which were lower than the values reported here. The perfusion rates of 93 ml./min. reported by these authors were much higher than the rates encountered in the present series of perfusions. Brauer *et al.* (1951) found that livers from male rats produced more bile than did livers from female rats. This was confirmed in the present series of perfusions, but when comparisons were made after correcting for differences in organ weights, the difference in bile production was not significant.

The oxygen requirements of the perfused liver in relation to blood flow and bile production.

The effect of hypoxia on the production of bile by the liver *in situ* has been studied by Engstrand (1949). In the dog, the bile flow decreased and finally stopped after a period of 30-50 min. breathing 9 p.c. oxygen. The secretion of bile ceased even though the portal pressure remained essentially unchanged during the period of hypoxia.

The consumption of oxygen by the liver has been estimated at 0·45 ml./10 gm. liver weight/min. for the dog (Blalock and Mason, 1936) and 0·5 ml./10 gm. liver weight/min. for the rat (D'Silva and Neil, 1954). The oxygen capacity of the perfusion fluid used in the experiments described in this paper was about 13 vols. p.c.; thus a flow rate of about 4 ml./min. would account for the oxygen requirements of a liver weighing 10 gm. This rate of flow would be adequate only if the liver was able to extract oxygen from the perfusate almost completely. The oxygen consumption of the liver has been shown to be reduced at low temperatures (Gray, Rueckert and Rink, 1956) so that the requirements of oxygen by livers perfused at 32° C. would be met by a lower rate of blood flow.

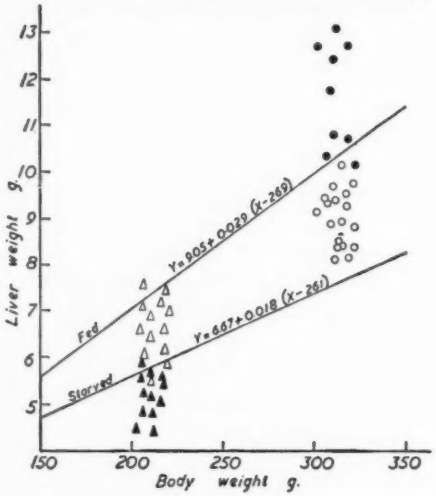

Fig. 7. *The effect of perfusion pressure on the final weight of the liver.* The two regression lines show the relationship between body weight and liver weight for fed rats and for rats starved 24 hr. The points shown on the graph represent perfusion experiments carried out at 38° C.

△ Livers from fed rats perfused at a pressure of 15 cm.

▲ Livers from starved rats perfused at a pressure of 15 cm.

● Livers from fed rats perfused at a pressure of 5 cm.

○ Livers from fed rats perfused at a pressure of 15 cm.

The flow of blood through livers perfused at a pressure of 5 cm. of blood was about 2-4 ml./10 gm. liver weight/min. After four hours' perfusion, the weights of these livers were significantly greater than the weights of normal livers (Fig. 7). When the perfusions were carried out at pressures of 15 or 25 cm. of blood, the weights of the livers were significantly less than normal (Fig. 7). This held for livers from both starved and fed rats. The rates of blood flow at pressures of 15 and 25 cm. were about 8 and 14 ml./10 gm. liver weight/min. respectively.

It appeared that in those perfusions carried out at a pressure of 5 cm. the supply of oxygen was inadequate to meet the requirements of the hepatic cells. This would be particularly so at temperatures of 42° C. The livers became grossly oedematous and bile production fell off rapidly. Apart from the effects that anoxia may have on the metabolic activity of the hepatic cells and the secretion of bile, the swelling of the liver tissue and the accumulation of oedema fluid within the relatively indistensible liver capsule may have the effect of mechanically obstructing the transfer of bile from the canaliculi to the collecting ducts. The excess water content of the liver did not appear to lead to any increased excretion of fluid in the bile. At perfusion pressures of 25 cm. of blood, the amount of bile secreted at 42° C. was only slightly less than at 38° C. It appeared that at 42° C. a flow of about 14 ml./10 gm. liver weight/min. was required to meet the oxygen requirements for maximal production of bile.

The effects of perfusion pressure and temperature on the production of bile by the perfused rat liver have been investigated by Brauer, Leong and Holloway (1954). These workers found that at perfusion pressures above 7-8 cm. of blood, the production of bile was unaffected by further increases in pressure. They also showed that as the temperature was lowered to 25° C. the production of bile fell off from a maximum at 40-41° C. In the present series of experiments, it was found that an increase in the perfusion pressure from 5-25 cm. of blood caused a significant increase in bile production. This different finding may be related to differences in blood flow and oxygen supply between the preparations. The rates of blood flow reported by Brauer *et al.* (1954) were about ten times higher than those found in the experiments described above. If the factors limiting bile production by the perfused liver were blood flow and oxygen supply, once all the liver sinuses were opened up and the oxygen requirements of all the hepatic cells were being met, bile production would reach a limiting value which would be altered only by changes in other factors concerned in the process of bile secretion. The results obtained in the experiments described in this paper may agree more closely with experiments carried out by Brauer and his colleagues below pressures of 7 cm. of blood.

It is difficult to find a reason for these large differences in flow rates. Brauer, Leong, Holloway, Krebs and Pessotti (1954) have concluded that the isolated liver is in a state of vaso-dilatation and that this accounted for the high rates of flow they found in the perfused organ. No evidence of this was found in the present series of perfusion experiments and flow rates at physiological pressures were close to those which occur in the intact liver.

Fat is known to have a choloretic and a cholagogic effect on the liver when it is given in a meal. The failure of changes in the concentration of chylomicron fat in the perfusate to produce any significant effect on bile production in the perfused liver indicated that these effects are not produced by the direct action of absorbed fat on the liver cells even though the liver cells are known to take

up large amounts of absorbed fat (cf. Morris and French, 1958). The mechanism would appear to be related to the presence of fat in the intestine itself prior to absorption.

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THE EFFECTS OF SUTURE MATERIALS AND SUTURE TECHNIQUES ON THE HEALING OF WOUNDS IN THE SKIN OF THE SHEEP

by A. K. LASCELLES[1] AND P. J. CLARINGBOLD[2]

(From the Faculty of Veterinary Science, University of Sydney, N.S.W.).

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SUMMARY.

Vetafil, braided polyester yarn, braided silk, monofilament nylon and plain catgut were used to suture wounds in the skin of sheep. The apposition of the edges of the wound, the gauge of the suture material, the tension of the sutures and the dressing were varied. The strength of the wound was used to assess the effect of these various factors. No significant differences were found with the exception that very closely spaced sutures caused a significant decrease in the strength of the wounds. The inflammatory response in the skin caused by the various suture materials was examined histologically. The response varied considerably, but no difference between the various suture materials could be seen.

INTRODUCTION.

The effects of various types of suture material on the healing of wounds has been studied histologically in animals by Madsen (1953a) and Meade and Ochsner (1940). These authors found that catgut caused a greater exudation and delay in the onset of fibroplasia than did the non-absorbable types of suture materials. Howes (1933) and Madsen (1953b) used the strength of the sutured wound as a measure of the degree of wound healing of wounds in the wall of the stomach and in the abdominal fascia. They measured the strength of the wound with the suture left *in situ* and thus their results do not measure the strength of the wound alone.

Borgström and Sandblom (1956) studied the rate of healing of skin incisions closed with 000 silk using the various suture techniques which are used in routine surgical practice. No significant effects were demonstrated in wounds made without tissue tension.

Lascelles and Manusu (1959) have demonstrated the suitability of the large skin surface of the sheep for factorially designed experiments on wound healing. Studies made in this way were found to be efficient and permitted the examina-

[1] Department of Veterinary Medicine, University of Sydney. Present address: Department of Experimental Pathology, John Curtin School of Medical Research, Canberra.
[2] Department of Veterinary Physiology, University of Sydney, N.S.W.

tion of factor interactions. A fractionally replicated factorial experiment has been used in the present study to examine the effects of several factors of surgical interest. This was done in two experiments, and some overlap of the factors examined was introduced to check the reliability of the results. In addition, the inflammatory response produced by the various suture materials was examined histologically.

MATERIALS AND METHODS.

Experiment 1.

Suture materials. Two suture materials were tested in this experiment; black braided polyester yarn (Johnson and Johnson) and Vetafil (Bengen). The latter material is commonly used in veterinary practice for suturing the skin. The square knot was used to tie the Vetafil and the standard nylon knot was used to tie the polyester yarn.

Gauge. Two different gauges of the suture materials were used; 000 and 0 polyester yarn and 0·2 mm. and 0·4 mm. Vetafil.

Apposition. Simple sutures were used to produce edge to edge apposition, while mattress sutures were used to exert the edges of the wound.

Continuity and spacing. The effect of continuity of suturing was examined by suturing the wounds with either continuous or interrupted sutures. The effect of spacing of the sutures was examined by comparing incisions closed with sutures spaced 0·75 cm. and 0·25 cm. apart.

Tension. The effect of tension of the sutures was examined by comparing wounds which were closed with just sufficient tension to the sutures to cause the edges to remain apposed with wounds in which the tension applied to the sutures was maximal, consistent with reasonable surgical technique.

Dressing. The effect of dressing wounds was examined by comparing wounds left exposed with wounds sealed aseptically with collodion. Four sheep were used in this experiment and the total number of treatment combinations was 512. As sixteen wounds were a reasonable number to study on each sheep the experiment was designed as a 1/8 replicate.

Experiment 2.

Suture materials. The suture materials used were black braided polyester yarn, black braided silk, monofilament nylon and plain catgut. These were obtained from Messrs. Johnson and Johnson Pty. Ltd.

Gauge. The fine and coarse gauges of the polyester yarn, silk and catgut were respectively 000 and 0 and "fine" and "medium" gauge monofilament nylon were used. The silk and catgut were tied with a square knot and the polyester yarn and monofilament nylon were tied with a standard nylon knot.

Spacing and apposition. The spacing of the sutures in this experiment was 0·5 and 1·0 cm. and the different types of apposition were effected in a similar way to that in Experiment 1. The thickness of a fold of skin was measured with a micrometer screw gauge. Three measurements were made along each wound site and averaged. Four sheep were used in this experiment and the total number of treatment combinations was thus 128. The experiment was designed as a 1/2 replicate.

The preparation of the sheep for surgery has been described previously (Lascelles and Manusu, 1959). One to two years old crossbred wethers were used in both experiments. The position of the treatment combination on the experimental animals and the order of operation were both at random. The wounds were removed from the animals seven days after the operation and the strength of each wound was measured (cf. Lascelles and Manusu, 1959). Small samples were taken from the centre of each wound and fixed in 12·5 p.c. W/V formal-saline. The sections were stained in haematoxylin and eosin and by van Gieson's method.

Experiment 3.

The inflammatory response caused by the various suture materials was examined histologically by inserting polyester yarn (gauge 0), braided silk (gauge 0), plain catgut (gauge 0), monofilament nylon (medium) and Vetafil (0·4 mm.) through a small pinch of skin using a No. 8 straight triangular-pointed needle. The knot used to tie each suture material has been described earlier. The experimental area was a closely shaved square on the flank of the sheep and the various suture materials were allotted by using a latin square design. Three sheep were used in this experiment. Samples of the sutured skin were removed after five days and fixed in 12·5 p.c. W/V formal-saline. The suture material was removed from each specimen prior to embedding. Serial sections were made and stained by haematoxylin and eosin and by van Gieson's method and the inflammatory reaction surrounding the suture track was examined.

RESULTS.

The analyses of variance of Experiments 1 and 2 are summarised in Tables 1 and 2. In the first experiment, the analysis shows that wounds closed with sutures spaced 0·75 cm. apart were stronger than wounds closed with sutures 0·25 cm. apart. The difference in strength was 17 p.c. and this finding is common to all sheep and is independent of other factors tested. Apart from the sheep variation observed, no differences in the other factors examined were seen.

TABLE 1.

Summary of the analysis of variance of the results in Experiment 1.

Source of variation	D.F.	Mean square strength of wound (Hectograms/cm)²
Main effects		
Suture material A Polyester yarn v Vetafil	1	0·14
Gauge B Fine v coarse	1	14·03
Apposition C Edge to edge v eversion	1	4·71
Continuity D Continuous v interrupted	1	4·87
Spacing E 0·75 cm. v 0·25 cm.	1	28·59**
Tension F Minimum v maximum	1	0·54
Dressing G Exposed v collodion	1	0·04
Unconfounded first order interactions	19	6·41
Sheep differences (Confounded with ABC, DE, FG interactions)	3	82·91***
Residual between wound error	34	4·68
Within wound error (measurement variance)	124	1·61
Missing values	4	—

** $0{\cdot}01 > P > 0{\cdot}001$.
*** $P < 0{\cdot}001$.

TABLE 2.

Summary of the analysis of variance of the results in Experiment 2.

Source of variation		D.F.	Corrected mean square wound strength (Hectograms/cm)²
Main effects			
Suture materials Polyester yarn v silk v monofilament nylon v catgut	A	3	2·60
Gauge Fine v coarse	B	1	0·55
Apposition Edge to edge v eversion	C	1	1·68
Spacing 0·5 cm. v 1·0 cm.	D	1	0·15
First order interactions		30	0·91
Sheep		3	110·64***
Residual between wound error (Measurement variance)		23	2·9
Covariance with thickness of skin		1	—

*** $P<0·001$.

No significant differences were found except between sheep in Experiment 2. Since there was no difference between wounds closed with sutures 0·5 and 1 cm. apart, a significant delay in wound healing will occur only when the sutures are very closely spaced as was the case in Experiment 1.

A significant correlation (0·439; $0·05>P>0·01$) between the strength of the wound and the thickness of the skin indicated that wound strength was influenced by the thickness of the skin. Correction of the strength of the wound for concomitant variation of skin thickness has led to an increase in precision in results as witnessed by the smaller error variance in Experiment 2.

Histology.

Experiments 1 *and* 2. Histologically, considerable variation in the degree of maturation of the fibroblasts was seen. The strength of the wound and the amount of collagen present were quite highly correlated (cf. Dunphy and Udupa, 1955).

Experiment 3. Examination of the inflammatory reaction caused by the various suture materials showed that great variation occurred in the degree of exudation, in the concentration and width of leucocytic infiltration, and in the extent and maturity of the surrounding fibroplasia. The inflammatory reactions were assessed qualitatively. No clear-cut differences in the reactions caused by the various sutures could be seen.

DISCUSSION.

Although a small but significant difference was found between wounds sutured with different degrees of spacing, the large variation in the strength of

wounds in different sheep makes this effect of no surgical consequence. The absence of significant differences between the variant suture techniques on the healing of the wounds is in agreement with Borgström *et al.* (1956). The type of suture material used was not found to influence the repair of the wounds. This finding is not compatible with the conclusions of Howes (1933) and Madsen (1953b) who based their findings on the strength of the wound with the suture left *in situ*. The results in these experiments do not agree with those of Preston (1940), who examined the effect of a number of suture materials used together with various suture techniques on the healing of wounds in the skin of the rat. Two incisions were made on each rat and since only one type of suture material appeared in each rat it is not possible to test the significance of the differences.

The failure to demonstrate any difference in the inflammatory response of the tissues around the various suture materials supports the above findings, although Madsen (1953a), Mahoney (1943), Meade and Ochsner (1940) and many others state that catgut causes the greatest inflammatory response in the tissues. It is possible that catgut causes less inflammation in the tissues of sheep and this may be due to the fact that catgut is made from the intestine of sheep.

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THE ACTION OF ANIONS ON CARDIAC MUSCLE

by W. G. NAYLER[1] AND M. McCULLOCH[2]

(From the Baker Medical Research Institute, Melbourne).

(*Accepted for publication 14th December, 1959.*)

SUMMARY

Substitution of chloride by bromide, nitrate and iodide lessened the force of isotonic contraction in cardiac muscle.

Toxic effects found during iodide perfusion were reversed by strophanthin-G, $CaCl_2$, plasma and fresh Ringer solution. Low $CaCl_2$ and high KCl precipitated toxicity.

Bromide, nitrate and iodide failed to affect contracture.

The contrast between these results and those reported for skeletal muscle is discussed.

INTRODUCTION.

Previous investigators have studied the action of anions on skeletal muscle. Kahn and Sandow (1950, 1951, 1955) confirmed the earlier reports of Schwartz (1907) and Chao (1935), concluding that the tension and duration of a twitch in isolated frog skeletal muscle was increased in the order $Br < NO_3 < I$ when these anions were substituted for chloride in Ringer solution. In 1954 Hill and MacPherson, also using frog's skeletal muscle, noted that heat production accompanying the twitch, as well as twitch tension, followed this pattern. During tetanic contraction, however, he found that the heat production, tension and velocity of shortening under any load were unaffected by these anion substitutions.

As yet the action of anions on the contractile behaviour of the myocardium has not been extensively studied. The experiments described here, which were carried out on toad ventricular muscle, indicate that cardiac and skeletal muscle may differ qualitatively from each other in their response to bromide, nitrate and iodide when these anions are substituted for the chloride normally present in the perfusion fluid.

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[2] Alfred Hospital Research Fellowship.

METHODS.

Isolated ventricles from winter toads (*Bufo marinus*) were used throughout these experiments which were carried out at room temperature (19-23° C.) using Ringer solution of the following composition:

NaCl	115 mM
$NaHCO_3$	20·6 mM
$NaH_2PO_4 2H_2O$	3·0 mM
$MgSO4.7H_2O$	1·2 mM
Glucose	16·5 mM
KCl	3·2 mM
$CaCl_2$	1·3 mM

Ninety-five p.c. O_2 and 5 p.c. CO_2 was continuously bubbled through the perfusate to ensure aerobic conditions. When the chloride of the Ringer solution was replaced by bromide, nitrate or iodide a constant sodium concentration was maintained, the anion substitution involving only the chloride of the sodium chloride.

Ventricular isotonic contractions were recorded photo-electrically, using a modified version of the apparatus described by Hajdu and Szent-Gyorgyi (1952). The basic features of this apparatus are shown in Fig. 1. A thin rigid wire with a bead at one end is introduced into a cannula (1 ml. capacity) placed in the aorta so that the bead rests against the endocardium. At the free end of the wire a light shutter lies directly between a constant light source and a photo-electric cell, so that during ventricular contraction and relaxation the shutter is displaced, and the light entering the P.E. cell is varied. The fluctuating voltage so produced is fed into a linear D.C. amplifier and recorded on a direct inking Evershed and Vignoles recorder running at 8 or 30 cm. per minute as required. This photo-electric method of recording the isotonic force of contraction obviates the errors introduced in classical methods, where the friction of the lever against the drum causes damping of the lever excursion.

When detailed records of the contraction-relaxation curves were required the recorder was run at the faster speed — see Fig. 2.

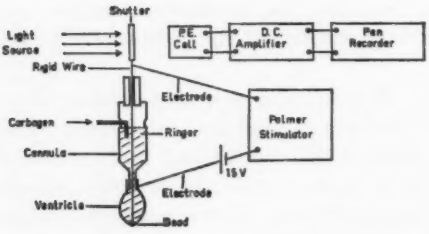

Fig. 1. Schematic diagram of apparatus.

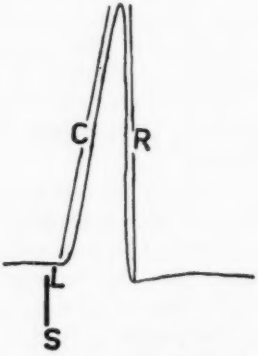

Fig. 2. A typical isotonic contraction. Recorder speed 30 cm./min.
C = contraction
L = latent period
R = relaxation
S = stimulus

During dissection the pacemaker was destroyed by cauterization. Electrodes were attached, one to the heart and the other to the shutter to form a circuit through a battery and Palmer type stimulator. In the experiments described here the ventricle was stimulated supramaximally at 30 beats/minute; a 15-minute equilibration period was allowed before observations were commenced.

This apparatus was similarly used to record the ventricular contractions of spontaneously beating hearts. A cannula was placed through the aorta as before, but the pacemaker was left intact.

In a series of experiments using both spontaneously beating and electrically stimulated ventricles, the chloride in the perfusate was replaced with bromide, nitrate or iodide and the isotonic force of contraction recorded both before and following the anion substitution. In those experiments in which an effect was not apparent immediately, observations were continued for at least thirty minutes, and in some instances for two hours.

In other experiments using spontaneously beating recycling hearts, the action of the above anions on the force of ventricular contraction was recorded. Both the conventional kymograph and the perfusion apparatus previously described (Nayler and McKelvie, 1956) were used to record the results. In all 97 experiments were performed.

Additional experiments were carried out to investigate the toxic effects usually noted during prolonged perfusion in iodide Ringer solution. The action of calcium and potassium ions, strophanthin-G and plasma (toad) on the iodide induced failure in stimulated preparations was studied.

Contracture. The effect of anions on the contracture produced in toad ventricular muscle by Ringer solution containing 100 mM KCl (Niedergerke, 1956) was also studied. Contractures induced in chloride Ringer solution were compared with those found in bromide, nitrate and iodide Ringer, records being made photo-electrically as previously described. 27 experiments were carried out.

RESULTS.

Replacement of chloride by bromide, nitrate and iodide in both the spontaneously beating and stimulated preparations failed to increase the force of contraction significantly—see Fig. 3 and Table 1 where these results are summarized.

Stimulated preparations.

During perfusion with bromide Ringer the contractile force was slightly reduced; with nitrate Ringer this reduction was more marked. Immediately following the addition of iodide Ringer, a slight transient increase in the force of contraction was recorded. In a high percentage of preparations this initial effect was followed by the onset of toxicity.

Spontaneous preparations.

In general, the observations made using spontaneously beating preparations confirmed the above results. The toxic effects of iodide were more pronounced usually leading to cardiac arrest.

Iodide toxicity.

Stimulated preparations. Fifty-five p.c. of stimulated preparations perfused in iodide Ringer solution developed toxic symptoms, the average time of onset

being twenty-five minutes. These toxic effects were characterized by the blocking of every second impulse (2:1 block)—see Fig. 4.

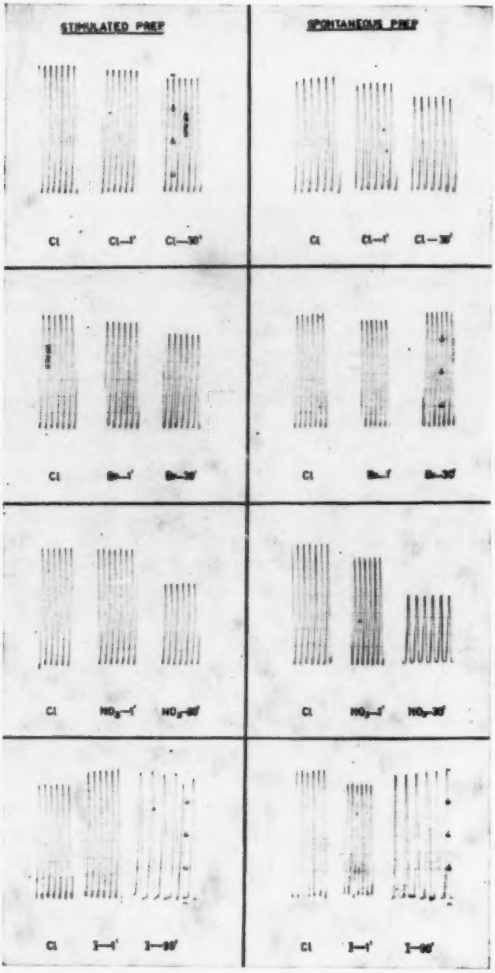

Fig. 3. Isotonic contractions in stimulated and spontaneously beating preparations, one minute and thirty minutes after perfusion with chloride, bromide, nitrate and iodide Ringer solutions. Recorder speed 8 cm./min.

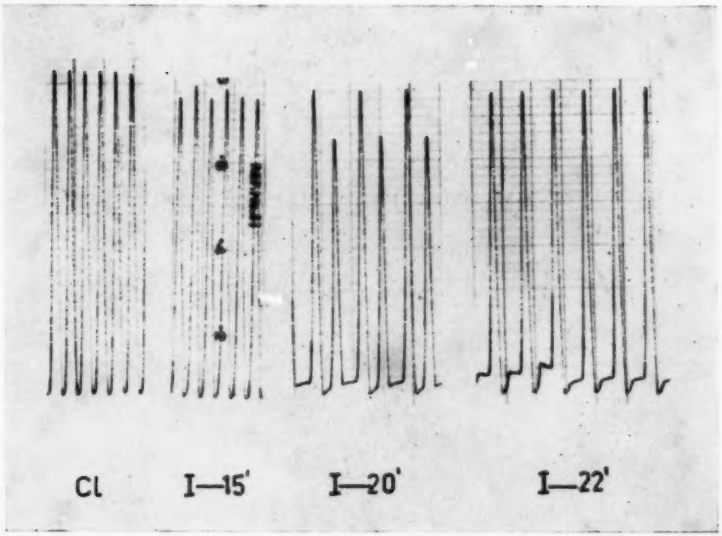

Fig. 4. Records taken fifteen, twenty and twenty-two minutes after commencing iodide perfusion of a heart initially perfused in chloride Ringer. Note onset of iodide toxicity (2:1 block). Recorder speed, 8 cm./min.

The effect of the following substances on the block when added to the iodide perfusion fluid was studied:

Substance.	*Response.*
0·67 mM KCl	Toxicity increased.
0·45 mM $CaCl_2$	Block immediately cleared.
25 μg. strophanthin-G[3] ...	Block immediately cleared.
0·1 ml. plasma (toad) ...	Block immediately cleared.
Fresh Ringer solution ...	Block immediately cleared.

These results are summarized in Fig. 5.

It was noted that hearts showing no conduction abnormalities in normal calcium iodide Ringer developed the characteristic 2:1 block when the calcium concentration was halved.

Spontaneous preparations. The toxic effects recorded in these preparations during iodide perfusion were more pronounced but followed the same pattern as those noted in the stimulated series above. The toxicity usually resulted in cardiac arrest.

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[3] Strophanthin-G as Ouabain Arnaud Laboratoire Nativelle product.

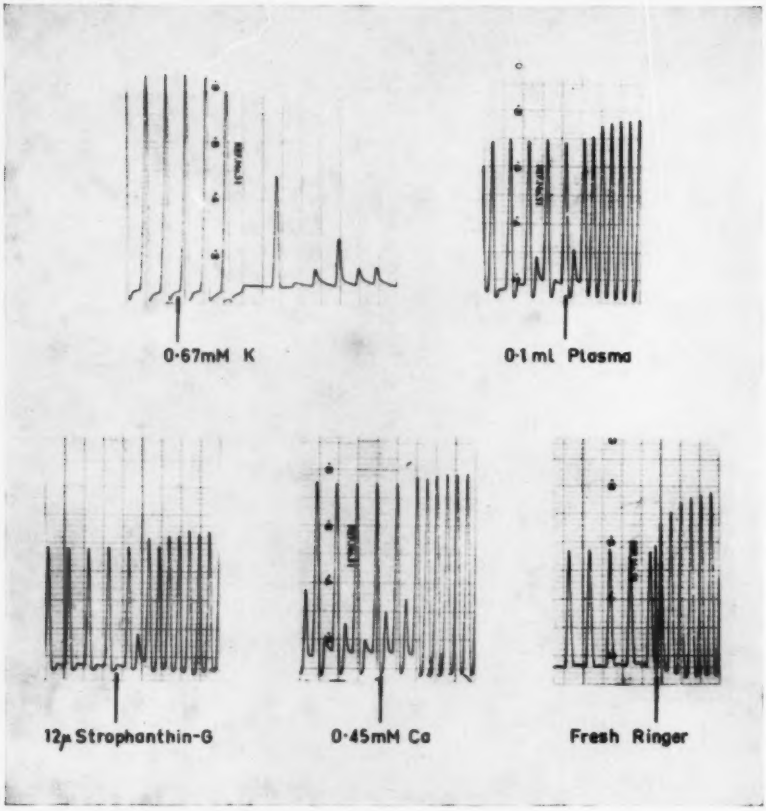

Fig. 5. Records showing action of 0·67 mM K (as KCl), 0·1 ml. plasma (toad), 12 μg. strophanthin-G, 0·45 mM Ca (as $CaCl_2$), and fresh Ringer solution on iodide toxicity. Recorder speed, 8 cm./min.

The effect of anions on contracture.

The substitution of chloride in the perfusate by bromide, nitrate or iodide had no marked effect on the contracture induced by 100 mM KCl solution. Contracture in bromide and nitrate was either lower or the same as that in chloride, whilst contracture in iodide solution was slightly higher. In other experiments the effect of plasma on contracture recorded in normal chloride Ringer solution was investigated. Although plasma slightly increased the height of contraction it failed to alter contracture—see Fig. 6.

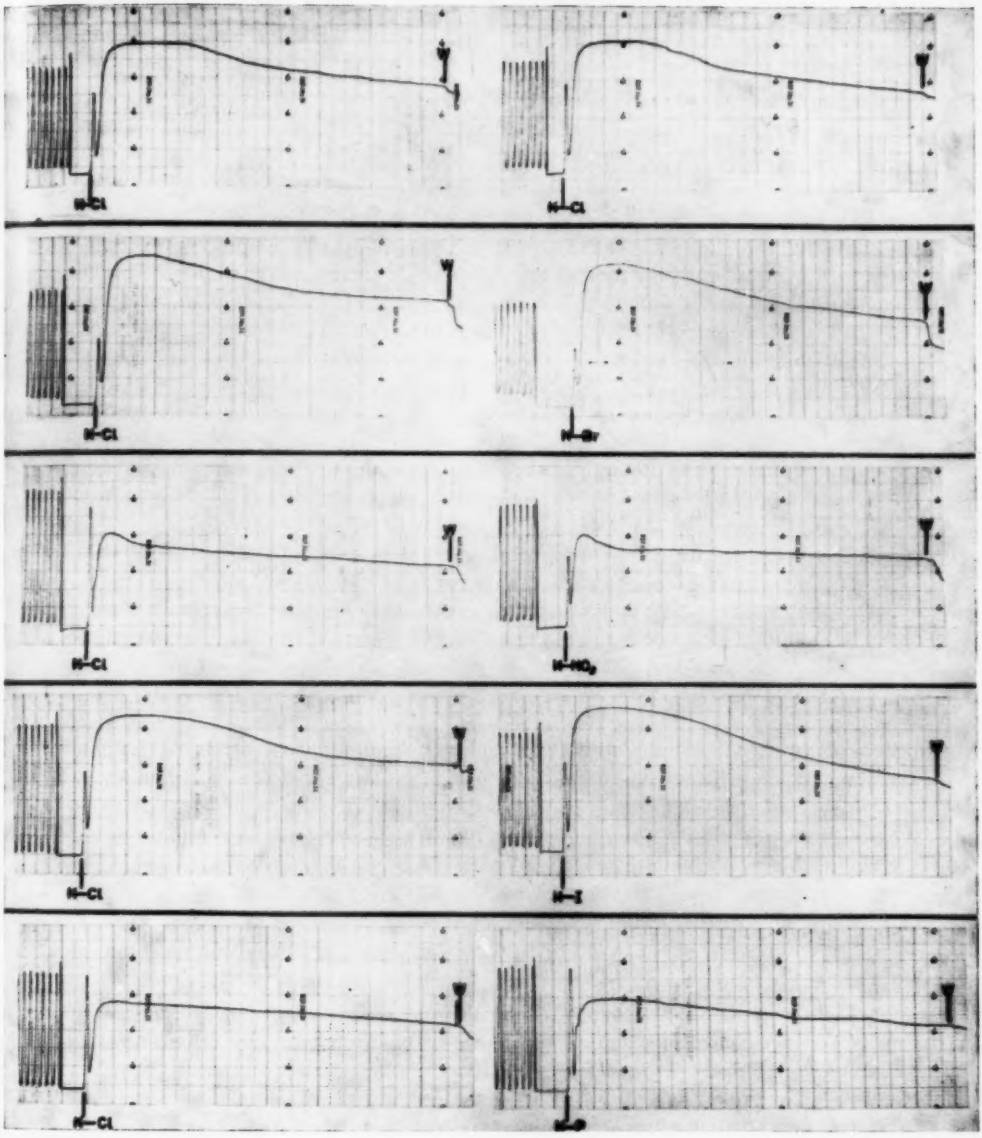

Fig. 6. Records comparing contracture induced by 100 mM KCl in chloride Ringer solution with that similarly produced in the anion substituted Ringers. Paired tracings from 5 hearts are shown.

K — Cl = 100 mM KCl in chloride Ringer
K — Br = 100 mM KCl in bromide Ringer
K — NO_3 = 100 mM KCl in nitrate Ringer
K — I = 100 mM KCl in iodide Ringer
K — P = 100 mM KCl in chloride Ringer containing 0·1 ml. plasma/ml.
W = wash.
Time interval between K and W = 3 mins.
Recorder speed, 8 cm./min.

TABLE 1.

Anion	Effect on contraction height			
	Stimulated preparation		Spontaneous preparation	
	No. of Exp.		No. of Exp.	
Chloride	6	14 p.c. reduction (4)* No effect (2)	6	10 p.c. reduction (3) No effect (3)
Bromide	6	17 p.c. reduction (5) No effect (1)	6	13 p.c. reduction (4) No effect (2)
Nitrate	12	32 p.c. reduction (9) No effect (3)	12	35 p.c. reduction (11) No effect (1)
Iodide	18	19 p.c. reduction (9) 15 p.c. increase (7) No effect (2) 10 developed 2:1 block in av. 25 mins. 8—no toxic effects in av. 50 mins.	10	15 p.c. reduction (9) No effect (1) 6 stopped in av. 7 mins. 3 slowed in av. 5 mins.

* The number of preparations in which that particular result was recorded is shown in brackets.

DISCUSSION.

Hill and MacPherson (1954), Kahn and Sandow (1950, 1951, 1955) and others have shown that the anions bromide, nitrate and iodide when used to replace the chloride of the perfusion fluid, increased the force of contraction in skeletal muscle. Hill and MacPherson have suggested that an increase in the active state of the muscle is associated with the increased tension observed. The results presented here indicate that cardiac muscle differs from skeletal muscle in its response to these anions. Thus, even after prolonged perfusion, replacement of chloride by them failed to produce any increase in the isotonic force of contraction.

The difference in the behaviour of skeletal and cardiac muscle during the above anion substitutions suggests that the mechanism whereby the contractile force is varied may differ in these preparations. Abbott and Mommaerts (1959) arrived at a similar conclusion during their study of force-velocity curves in skeletal and cardiac muscle. In contrast to the data already available for skeletal muscle they found that the "plateau of full activity" was unaltered or shortened during a positive inotropic response in cardiac muscle strips.

The literature contains few references to the effect of anions on cardiac muscle. Clark (1913) observed that anions had no beneficial effect on the hypodynamic frog's heart. Hutter and Noble (1959) using Purkinje fibres from

dog's and sheep's hearts recorded disturbed conduction during perfusion with solutions containing bromide, nitrate and iodide.

In skeletal muscle Hill and McPherson (1954) noted that continued perfusion in 100 p.c. iodide-Ringer produced irreversible toxic effects. During our experiments using cardiac muscle, toxic effects became apparent during iodide perfusion, but these could be reversed by the addition of strophanthin-G, plasma or $CaCl_2$. Since reduction of calcium in the iodide perfusate accelerated the onset of toxic symptoms, it can be postulated that iodide toxicity is associated with the formation of an "iodide calcium complex". It is noteworthy that in skeletal muscle there is evidence to suggest that calcium may act as the link between the action potential and the contraction. Bianchi and Shanes (1958) have shown an increased rate of calcium influx in skeletal muscle during nitrate perfusion. This may be the mechanism whereby an increased twitch tension is produced in skeletal muscle when nitrate is used to replace the chloride of the perfusate. These workers have linked this increase in calcium influx with the fact that nitrate increased potassium contracture. Such information is not as yet available for cardiac muscle. If this hypothesis is correct our experiments indicate that replacement of chloride by other anions has failed to alter significantly the rate of calcium influx in cardiac muscle. Hence, contractures recorded in bromide, nitrate and chloride Ringer solutions show no appreciable difference. It seems doubtful whether the slight transitory increase in contracture recorded during iodide perfusion could be due to increased calcium influx, since the data obtained during the investigation of iodide toxicity suggest that iodide in some way decreases the availability of the calcium in the perfusate to the heart. It is possible that the ability of strophanthin-G to reverse the iodide toxicity is linked with the action of this drug on the localisation of calcium at the cell membrane (Niedergerke, 1956). It will be recalled that this effect of strophanthin-G was immediate, pointing to a direct surface action. Similarly, the effect of plasma and $CaCl_2$ were instantaneous.

Our results imply that the anions play only a minor part in regulating cardiac activity. It is hoped that further studies using an isometric recording device will provide more information relating to the apparent differences between cardiac and skeletal muscle observed in this study.

Acknowledgments. We wish to thank Dr. T. E. Lowe for his stimulating discussions, and Mr. J. L. Bremner for his technical assistance.

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THE POSITIVE INOTROPIC ACTIVITY OF PLASMA

by W. G. NAYLER[1] AND M. McCULLOCH[2]

(From the Baker Medical Research Institute, Melbourne).

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SUMMARY.

Plasma exerts a positive inotropic action on the isolated heart. The inotropic response is similar to that induced by the glycosides.

DNP and quinidine sulphate both fail to depress the activity of plasma but abolish that of the glycosides.

Plasma activity is increased when hearts are perfused in low calcium Ringer solution.

Anterior pituitary extract restored the response of the winter heart to plasma.

The possibility that plasma normally contains a substance which regulates myocardial contractility is discussed.

INTRODUCTION.

In 1952 Hajdu and Szent-Gyorgyi confirmed and extended the reports of earlier workers describing the existence of a cardioactive substance in serum. Bowditch (1872), Ringer (1882), and Clark (1913) noted that the hypodynamic state induced in isolated hearts by prolonged perfusion in a protein free salt solution could be reversed by substituting serum for the perfusion fluid; Clark suggested that a lipid factor acting at the cell surface was involved in this stimulant activity. However, Hajdu, Weiss and Titus (1957), following Hajdu and Szent-Gyorgyi's earlier study on the action of serum and other agents on the classical staircase phenomenon, have recently proposed that a factor with digitalis-like properties rather than a lipid fraction is involved. Both serum and digitalis abolished the staircase whereas the lipids failed to do so.

The experiments described here were planned to investigate further the cardioactive properties of plasma and to determine how closely the plasma response resembles that recorded during glycoside activity under identical conditions. Plasma rather than serum was used in order to avoid the introduction of non-physiological compounds liberated during the clotting process. Toad plasma was added to isolated toad hearts so that problems associated with species specificity would not lead to erroneous results.

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[1] Part of the expenses of this investigation was defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

[2] Alfred Hospital Research Fellowship.

METHODS.

The perfusion apparatus and experimental procedure previously used to study the response of the isolated spontaneously beating heart to strophanthin-G, lanatoside C, and digitalis were used throughout these experiments without modification (Nayler and McKelvie, 1956). Perfusions were made at 25·0 ± 0·05° C. and, unless otherwise stated, perfusion fluid of the following composition used:

NaCl	115 mM
$NaHCO_3$	20·6 mM
$NaH_2PO_4.2H_2O$	3·0 mM
$MgSO_4.7H_2O$	1·2 mM
Glucose	16·5 mM
KCl	3·2 mM
$CaCl_2$	1·3 mM

Hearts removed from freshly pithed toads (*Bufo marinus*) were allowed thirty minutes' temperature equilibration at 25·0° C. before observations were commenced, and a further one hour's control perfusion prior to the addition of the plasma. Since the experiments extended from January to September both summer and winter toads were used.

During collection of the blood and separation of plasma, care was taken to avoid haemolysis, and only free flowing blood samples were used. Minimal quantities of heparin[3] were used to prevent clotting.

When assaying the activity of plasma, we endeavoured to use the isolated heart and plasma from the same toad.

Summer preparations.

In six experiments, following the preliminary hour's perfusion, 0·5 ml. plasma separated from freshly collected blood was added to the perfusion system, and the effects on rate of beating, maximum aortic pressure, drops per beat, oxygen uptake, and the E.C.G. were recorded throughout the ensuing two hours. Observations were made at fifteen-minute intervals except during that period immediately following the addition of the plasma, when three consecutive five-minute records were made. In an additional six experiments 0·75 ml. plasma was added as above, and the results of both series compared with those obtained from earlier studies in which the effect of strophanthin-G[4] (1×10^{-5} final conc.), lanatoside C[5] (2×10^{-5} final conc.) and digoxin[6] (1×10^{-6} final conc.) was similarly investigated (Nayler, 1956, 1957a). In other experiments strophanthin-G (final conc. 1×10^{-5}) was initially added to the perfusate, and the response to 0·5 ml. plasma recorded.

Because of the apparent importance of Ca++ in the functional activity of cardiac muscle (Niedergerke, 1956) a further series of six experiments was made in which 0·5 ml. plasma was added to hearts perfused with Ringer solution containing half normal calcium (0·65 mM). These results were confirmed in other experiments using the standard kymograph record of ventricular contractile force as the index of response, and compared with those previously noted using normal calcium concentration (1·3 mM).

Previous experiments have shown that preliminary perfusion of hearts with quinidine sulphate[7] (1×10^{-5} final conc.) abolished the positive inotropic activity of strophanthin-G (Nayler, 1958). Thus six experiments were performed in which 0·75 ml. plasma was added

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[3] Heparin: Boots Pure Drug Co.
[4] Strophanthin-G: Ouabain Arnaud, Laboratoire Nativelle Product.
[5] Lanatoside C: Cedilanide Sandoz Ltd. Product.
[6] Digoxin: Burroughs Wellcome & Co. Product.
[7] Quinidine Sulphate as Quinicardine, Laboratoire Nativelle Product.

to hearts previously perfused with quinidine sulphate enriched Ringer, and observations of rate of beating, maximum aortic pressure, drops per beat and rate of oxygen uptake were made as above. In other experiments the action of 0·5 ml. plasma on the ventricular contractile force of hearts perfused with dinitrophenol (DNP) enriched Ringer solution was determined. Previous experiments using DNP-treated hearts have shown that this drug, like quinidine sulphate, abolishes the positive inotropic activity of strophanthin-G (Nayler, 1957b).

Winter preparations.

Isolated toad hearts display seasonal variation in the magnitude of their response to the glycosides, a reduced sensitivity being recorded throughout the winter months. Accordingly, some experiments were carried out to determine whether or not this seasonal variation extended to the positive inotropic activity of plasma.

0·5 and 0·75 ml. plasma from winter toads was added to the isolated hearts, and the effects on rate of beating, maximum aortic pressure, drops per beat and E.C.G. were recorded as above. Since it was previously noted that insulin (5 units/litre) partially restored the positive inotropic activity of the glycosides on winter hearts (Nayler, 1957c), insulin[8] was added in a further series and the response to plasma tested as above. The activity of plasma removed from winter toads which had been injected 2-5 days previously with anterior pituitary extract[9] (0·5 gm.) was similarly studied, the plasma being added to both anterior pituitary treated and to normal winter hearts. In other experiments toads were injected for six days with 100 µg. thyroxine,[10] and the inotropic activity of their plasma investigated.

RESULTS.

Summer hearts.

Qualitatively, the positive inotropic response recorded following the addition of 0·5 or 0·75 ml. of plasma to the perfusate was identical with that recorded during glycoside activity (Nayler, 1956).

The plasma response, summarized in Fig. 1, was characterized by a sustained increase in maximum aortic pressure, increased output per beat and a slight increase in beat rate. These changes were associated with an augmented total work output and an overall increase in efficiency. The E.C.G. tracings showed changes in the 'ST' segment – see Fig. 2.

The magnitude of the plasma induced inotropic response varied with the calcium concentration in the perfusate, 0·5 ml. plasma exerting a greater action when the calcium concentration was reduced from 1·3 to 0·65 mM – see Fig. 3. As can be seen in Fig. 4, the kymograph studies confirmed this plasma-low calcium synergism.

Catechol amines apparently play no part in the response of the isolated heart to plasma, since reserpinised[11] hearts responded normally both to plasma

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[8] Insulin: Boots Pure Drug Co.
[9] Anterior pituitary extract: (Ambinon) Organon Laboratories.
[10] Thyroxine: as sodium thyroxine.
[11] Toads injected with Reserpine 3 days before use. Reserpine used as Serpasil: Ciba Product.

from untreated and from reserpinised toads – see Fig. 5, where 0·5 ml. plasma from a reserpinised toad was added to the heart isolated from that toad.

Whereas the positive inotropic activity of strophanthin-G (Nayler, 1957, 1958) was abolished by pre-treatment of the isolated hearts with quinidine sulphate or DNP in the concentrations used above, a normal inotropic response was recorded following the addition of plasma to these hearts—Figs. 6, 7. Neither DNP nor quinidine sulphate interfered with the positive inotropic activity of plasma.

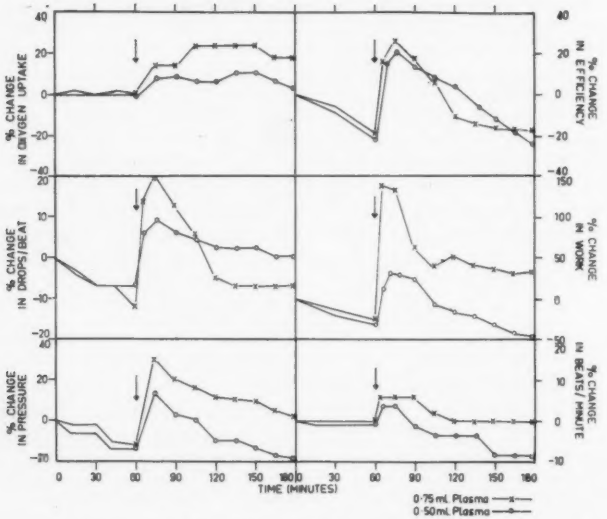

Fig. 1. Effect of 0·5 and 0·75 ml. plasma on the work output, efficiency, beat rate, drops per beat, maximum aortic pressure and oxygen uptake of the isolated heart. Plasma was added at the arrow. Each curve represents the mean of six experiments.

The addition of 0·5 ml. plasma to a series of hearts perfused with strophanthin-G enriched perfusate (final conc. 1×10^{-5}) resulted in a normal positive inotropic response which could not be distinguished from that recorded following the action of plasma on normal hearts – see Fig. 8. These graphs show no evidence of competition between plasma and strophanthin-G for sites of action.

Winter hearts.

During winter months the inotropic activity of plasma was absent or markedly reduced. During late August and early September the plasma activity, when tested on untreated hearts, returned slowly. Injection of the winter toads with thyroxine, and the addition of insulin to the perfusate both failed to restore the plasma response.

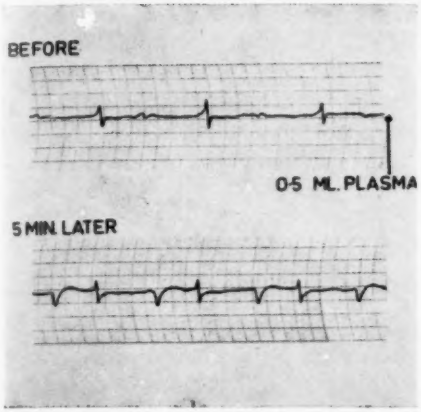

Fig. 2. E.C.G. tracing from perfused heart immediately before and five minutes following the addition of 0·5 ml. plasma.

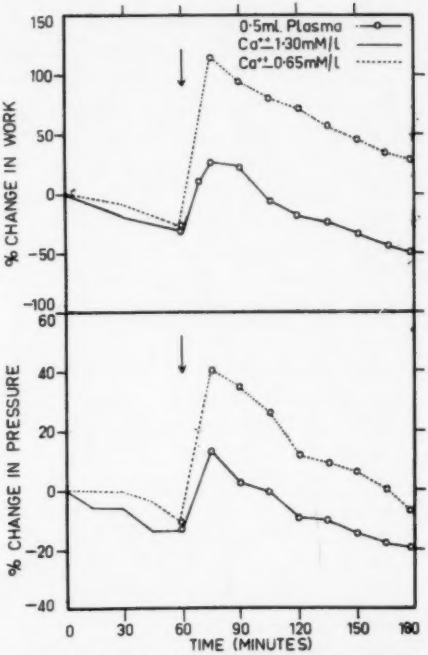

Fig. 3. Effect of 0·5 ml. plasma on the work output and maximum aortic pressure of hearts perfused in Ringer solution containing 1·3 mM or 0·65 mM calcium. Plasma was added at the arrow. In both cases the mean results of six experiments are displayed.

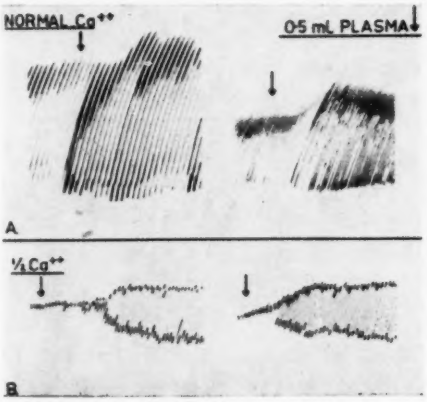

Fig. 4. Kymograph tracings showing the effect of 0·5 ml. plasma on the ventricular contractile force of two hearts perfused in Ringer solution containing normal (1·3 mM) calcium, and two other hearts perfused in half normal (0·65 mM) calcium. Plasma was added at the arrows.

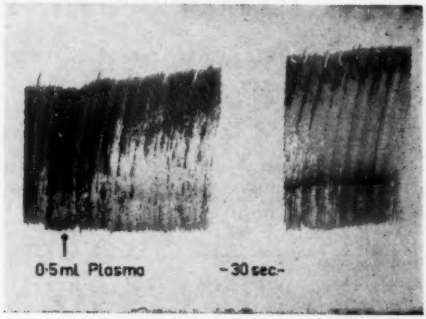

Fig. 5. Kymograph tracing showing the effect of 0·5 ml. plasma from a reserpinised toad on the ventricular contractile force of the heart removed from that toad.

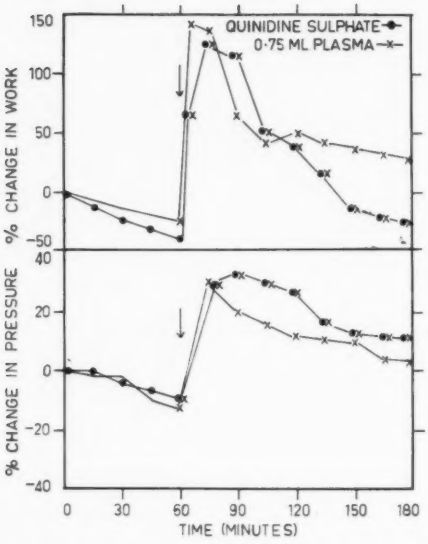

Fig. 6. Results showing the effects of quinidine sulphate (1×10^{-5} final conc.) on the increased aortic pressure and work output normally recorded following the addition of 0·75 ml. plasma. In each case the mean results of six experiments are displayed.

However, plasma from toads which received anterior pituitary extract injections displayed positive inotropic activity when tested on normal winter hearts and on hearts removed from anterior pituitary treated toads. Similarly plasma from normal winter toads showed slight activity when tested on hearts removed from anterior pituitary treated toads.

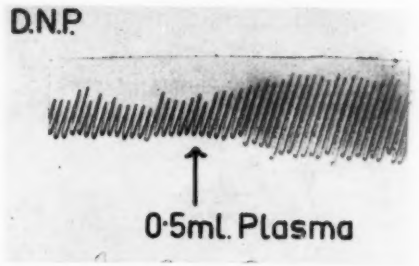

Fig. 7. Kymograph tracing showing the effect of DNP ($1{\cdot}09 \times 10^{-5}$ M) on the response of the isolated heart to plasma — 0·5 ml. added at the arrow.

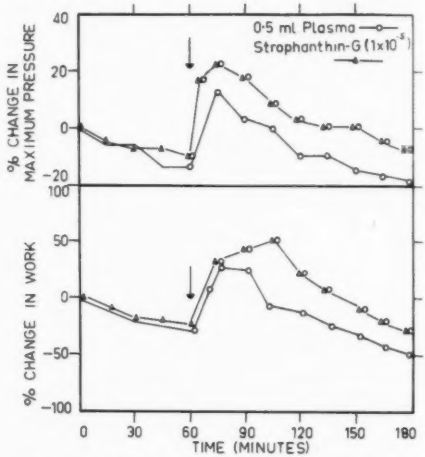

Fig. 8. Effect of the previous administration of strophanthin-G (1×10^{-5}) on the increased work output and maximum aortic pressure normally recorded following the addition of 0·5 ml. plasma. Each curve represents the mean results of six experiments and plasma was added at the arrow.

DISCUSSION.

These results demonstrate that although the positive inotropic response recorded following the addition of plasma to the perfused hypodynamic heart resembles that produced by the glycoside, these responses are not identical as would be expected if the plasma factor was a "digitalis-like substance" (Hajdu, Weiss and Titus, 1957).

Increased maximum aortic pressure and cardiac output (as indicated by number of drops per beat), augmented oxidative metabolism and an overall higher level of efficiency are features common to both responses. Two main differences emerge from this study – firstly, quinidine sulphate and DNP at concentrations which inhibit the positive inotropic activity of the glycosides (Nayler, 1957b, 1958) failed to inhibit that due to plasma. Secondly, the plasma response is evident within several minutes whereas approximately 60 minutes elapse before the maximum response to the glycosides is recorded in these preparations. These differences suggest that plasma and the glycoside, though acting on the same general system, are acting at different points in a chain of events. The rapidity with which the plasma effect is recorded suggests that a surface binding mechanism may be involved.

There is now considerable evidence to support the hypothesis that calcium binding at the cell membrane plays a part in regulating the contractile response (Bianchi and Shanes, 1958, 1959; Niedergerke, 1956; Shanes and Bianchi, 1959). The increased response recorded in this series following plasma activity in low calcium Ringer raises the possibility that plasma contains a factor which regulates the Ca^{++} binding capacity of the membrane and so, to some extent, regulates the activity of the myocardium. The activity of plasma cannot be explained simply in terms of changed potassium permeability, since pretreatment with quinidine sulphate failed to alter the response significantly (Armitage, 1957). Moreover, an increased supply of high energy phosphate bonds cannot be proposed to explain the inotropic activity of plasma since, unlike the glycosides, DNP failed to depress the response (Ellis, 1953; Wollenberger and Karsh, 1952; Nayler, 1957b).

Another point emerges from these results – that the activity of the plasma shows seasonal variation and therefore may be under hormonal control. This raises the possibility that plasma normally contains a substance whose function it is to regulate the activity of the myocardium possibly through a calcium binding mechanism. Since anterior pituitary extract partially restored the response of the winter hearts it may be that a pituitary secretion is involved in the response. No evidence was obtained to support any thyroid mechanism.

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HAEM SYNTHESIS *IN VITRO*

STUDIES WITH MAMMALIAN AND AVIAN ERYTHROCYTES

by PEGGY CLARK[1] AND R. J. WALSH

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

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SUMMARY.

The uptake of radioiron by mammalian reticulocytes is a rapid process and haem is synthesised from the iron almost immediately. The process is slower with avian erythrocytes and this is possibly related to a slower but more prolonged synthesis of protoporphyrin by the nucleated cells. This synthesis must account for all the Fe^{59} haemoglobin formed *in vitro* under the experimental conditions. Incubation of both reticulocytes and nucleated cells, before addition of iron, reduces both uptake of iron and synthesis of haem. It is postulated that there are two stages in the process of intracellular haem synthesis. The first is the entry of iron into the cell and this is inhibited by mercuric chloride but not by other enzyme inhibitors. The second phase, haem synthesis, is inhibited by potassium cyanide, and is therefore apparently dependent on oxidative respiration.

INTRODUCTION.

Mammalian reticulocytes take up iron from plasma and synthesise haemoglobin in the circulating blood, and this phenomenon has been demonstrated *in vitro* (Walsh *et al.*, 1949). Mature erythrocytes do not behave in the same manner, but it is not known whether this is a failure only of uptake or whether the mature red cell has lost its mechanism for haem synthesis. Erythrocytes from ducks (Jensen *et al.*, 1953) and from pigeons (Nakao, Plzak and Bethard, 1954) also accept iron and form haemoglobin *in vitro*.

In an earlier paper Clark and Walsh (1959) advanced the hypothesis that two phases were involved in this type of haemoglobin synthesis. They showed that the first phase, the entry of iron into the cell, was inhibited by mercuric chloride but not by potassium cyanide; whilst the second, the synthesis of haemoglobin, was inhibited by potassium cyanide but not by mercuric chloride. The nature of the non-haem iron in the cell was not investigated and its importance in the synthesis of haemoglobin was not established.

This paper reports the results of further studies with Fe^{59} on the kinetics of iron uptake and haem synthesis in rat, pigeon and human reticulocytes *in vitro* and on the effect of certain metabolic inhibitors on the uptake pattern.

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MATERIALS AND METHODS.

Reticulocytes.

No attempts were made to separate reticulocytes from mature red cells. A reticulocytosis was produced in laboratory rats by intraperitoneal injection of 0·9 to 1·0 ml. of 2 p.c. phenylhydrazine hydrochloride solution. Five days later the animals were anaesthetised and blood removed from the hearts after the chest was opened in the mid-line. Reticulocytosis was also produced in rats by removing approximately 5 ml. of blood by cardiac puncture. Samples containing reticulocytes were obtained three or four days later.

In every instance the blood samples were mixed with a solution containing 2·6 gm. of sodium citrate ($Na_3C_6H_5O_7.2H_2O$) 80 mg. of calcium penicillin and 330 mg. streptomycin hydrochloride per 100 ml. The amount used was approximately 1 ml. for each 4 ml. of blood.

Human blood containing reticulocytes was collected into the same solution from patients suffering from acquired haemolytic anaemia, congenital haemolytic anaemia and pernicious anaemia.

Avian erythrocytes.

Blood was obtained by direct cardiac puncture of pigeons and collected into the anticoagulant-antibiotic solution.

Bone marrow.

Bone marrow was obtained from the femora of rats. The animals were sacrificed, and loose tissue and muscle removed from the bones as soon as possible. The marrow tissue was removed by drilling holes through either end of the bone and forcing the anticoagulant solution into one hole with a syringe.

Radioactive iron.

$Fe^{59}Cl_3$ of high specific activity was obtained from Abbott's Laboratories (Chicago, U.S.A.) through the agency of the Commonwealth X-Ray and Radium Laboratory, Melbourne. It was diluted with physiological saline solution so that approximately 5 μc. were present in 1 ml. of solution.

Incubation of blood.

Aliquots of 1 ml. of blood were placed in 7-ml. vaccine vials which were stoppered with rubber bungs and rotated three times a minute in an incubator at 37° C. If the experiment exceeded six hours in duration the bungs were removed periodically for a few minutes to allow exchange of air. Test solutions and radioactive iron solutions (0·1 ml. to 1 ml. of blood) were added as required. At the end of each experiment the cells were washed ten times with approximately 6 ml. of physiological saline solution each time, and were finally haemolysed with distilled water in a plastic container. The volume was made up to 10 ml. and the radioassay made with an "EKCO" well-type scintillation counter.

Haem extraction.

The method described by Heilmeyer (1943) was used. The 10 ml. haemolysate were mixed with 60 ml. of glacial acetic acid and 200 ml. of ether. The whole was shaken in a separating funnel and extracted with water until the phases separated rapidly into a clear brown ether solution and a colourless water solution. The water extract contained iron which was designated "non-haem iron". The residual brown ether solution was extracted with 10 p.c. sodium hydroxide until the ether extract was colourless, and the iron in this extract was designated the "haem fraction". The distribution of haem and non-haem iron was confirmed by a modification of Fischer's method (1941) adapted for small quantities of blood.

Measurement of respiration.

Respiration measurements were made by the Warburg respirometer technique. The inhibitor was added to 1·5 ml. of blood. 0·2 ml. of 10 p.c. potassium hydroxide was placed in the centre well, except when cyanide was under test, when the mixture was equal parts of N.potassium cyanide and N.potassium hydroxide.

RESULTS.

Uptake of iron.

Blood samples were obtained from rats, pigeons and humans as described. $Fe^{59}Cl_3$ was added to each bottle (0·1 ml. containing approximately 0·5 μc. to 1 ml. of blood) and the bottles were incubated for varying periods. After the cells were washed and haemolysed the total uptake of iron was measured and the haem and non-haem fractions prepared. The results are shown in Tables 1 and 2, and in Figs. 1 and 2.

In the first experiment (Fig. 1a) with phenylhydrazine-induced reticulocytosis in rats, the total uptake at two hours was 82 p.c. of that at eight hours; the percentage of the iron in the haem fraction remained fairly constant at the three different times, averaging approximately 67 p.c. The experiment was repeated with haemorrhage-induced reticulocytes, and the results did not differ significantly from the pattern shown in Table 1 and Fig. 1a. The uptake of iron and the synthesis of haem were therefore rapid processes, and this was demonstrated further when the rate was measured at shorter intervals (Table 1 and Fig. 1b). The uptake at 30 minutes was 76 p.c. of that at three hours, with a progressive but small increase in both the total uptake and the percentage in the haem fraction. The experiment with rat bone marrow was continued for six hours. The total uptake of Fe^{59} was small, but maximum values were reached within four hours.

TABLE 1.

Uptake of iron at different times.

Experiment	Time of incubation (hours)	Number of samples	Mean total c.p.m.	Mean c.p.m. in haem fraction	Mean p.c. Fe^{59} in haem
Rat blood: phenylhydrazine-induced reticulocytosis	2	4	44,916	29,959	66·7
	4	4	48,681	30,182	62·0
	8	4	54,885	36,773	67·0
Rat blood: phenylhydrazine-induced reticulocytosis	1/2	4	36,582	24,327	66·5
	1	4	44,233	29,526	67·0
	2	4	48,947	33,896	69·0
	3	4	48,328	35,038	72·0
Pigeon erythrocytes	2	2	3,513	1,279	36·4
	4	2	7,707	3,306	42·9
	6	2	10,275	3,822	37·2
	24	2	15,475	4,472	28·9

TABLE 2.

Uptake of iron by human reticulocytes.

Blood samples from:	Mean c.p.m. uptake after incubation			
	2 hours	4 hours	6 hours	8 hours
Acquired haemolytic anaemia R.B.C.—$3 \cdot 3 \times 10^6$ reticulocytes 1·9 p.c.	813	1008	1239	—
Pernicious anaemia after vitamin B_{12} R.B.C.—$2 \cdot 1 \times 10^6$ reticulocytes 8·0 p.c.	1630	2104	1840	—
Acquired haemolytic anaemia. Hydrocortisone therapy R.B.C.—$3 \cdot 45 \times 10^6$ reticulocytes 5·8 p.c.	5170	9319	—	12,354
Congenital haemolytic anaemia reticulocytes 5·1 p.c.	428	745	—	920

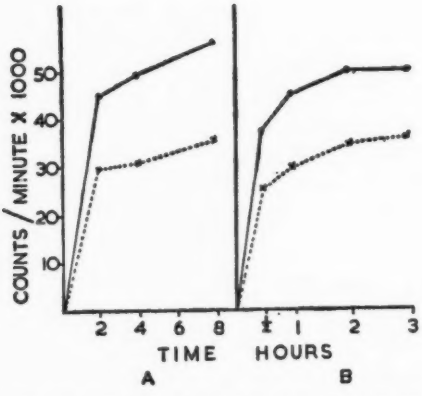

Fig. 1. The uptake of Fe^{59} and synthesis of haem by phenylhydrazine-induced reticulocytes in rats. A: during an 8-hour period; B: during a 3-hour period.

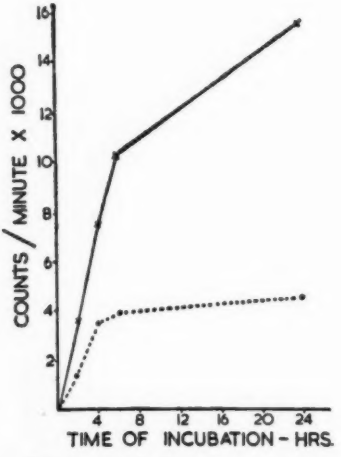

Fig. 2. Uptake of Fe^{59} and synthesis of haem by pigeon nucleated red cells.

Pigeon erythrocytes (Table 1 and Fig. 2) behaved in a different manner. The uptake was much slower and was progressive for at least 24 hours. The uptake was linear for the first six hours, but declined after this period. The possibility that the substrate was depleted at the later time intervals cannot be ignored. It should be noted that the percentage of Fe^{59} in the haem fraction, varying between 28 and 42·9 p.c., is lower than in the experiments with rat blood.

The blood of four human patients was investigated and the details and results are summarised in Table 2. There was no correlation between the number of reticulocytes in the specimen and the total uptake, and the rate of uptake varied in the different samples.

Effect of incubation on sample.

In this series of experiments the blood samples were incubated at 37° C. for various times before the addition of iron. A typical result with rat reticulocytes is shown in Fig. 3. There was a progressive reduction in the amount of iron entering the cell and this was associated with a reduction in the percentage of iron found in the haem fraction. It can be seen that the uptake of Fe^{59} (Table 3) was reduced from 10,985 c.p.m. to 1,070 c.p.m. after 24 hours' pre-

TABLE 3.

Effect of prior incubation on Fe^{59} uptake and incorporation into haem.

Time of prior incubation	Total counts	Counts in haem fraction	p.c. in haem fraction
0 hours (3 values)	10,985	5,405	49·2
2 hours (1 value)	8,551	3,566	41·7
4 hours (2 values)	6,335	2,091	33·0
6 hours (2 values)	4,566	1,671	36·6
24 hours (2 values)	1,070	308	28·8

liminary incubation, and the percentage in the haem fraction from 49·2 to 28·8. The percentage of haem iron in the control sample was less than in the experiment shown in Fig. 1 and in other experiments; this is due to one atypical value in the present series.

A similar experiment was performed with pigeon erythrocytes, but somewhat equivocal results were obtained with three different samples. One showed no significant change after 12 hours in either the total uptake or the percentage in the haem fraction; subsequently, the total uptake increased slightly and was highest following 24 hours' incubation. The other two samples showed an early increase in the total uptake but a considerable and significant reduction after 24 hours' incubation. In both samples the percentage in the haem fraction was reduced at 24 hours.

The possibility that glucose, inosine and inosine triphosphate would reduce or eliminate the effect of pre-incubation on rat reticulocytes was investigated. These substances have been shown to increase the survival time of stored blood by supplying substrate material. Glucose was used in a concentration of 1·8 p.c., and 0·3 ml. was added to 1 ml. of blood. Inosine and inosine triphosphate were added to the blood sample so that the concentration was approximately 10 μ.moles per ml. of blood. These substances did not alter the effect of pre-incubation on the rate of Fe^{59} uptake by the reticulocytes.

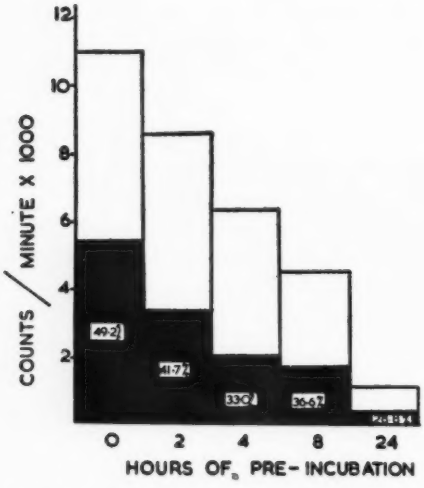

Fig. 3. Effect of preliminary incubation of rat reticulocytes on the subsequent uptake of Fe^{59} and haem synthesis. The solid portions of the columns represent Fe^{59} in the haem fraction.

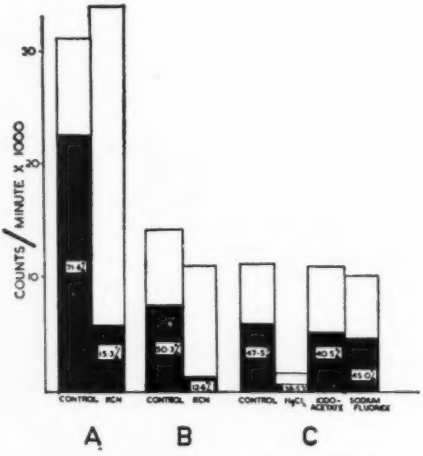

Fig. 4. The effect of metabolic inhibitors on Fe^{59} uptake and haem synthesis of phenylhydrazine-induced rat reticulocytes. The solid portions of the columns represent Fe^{59} in the haem fraction. The numbers of reticulocytes varied in the different samples. Experiments A and C were performed with phenylhydrazine-induced reticulocytes; Experiment B was performed with haemorrhage-induced reticulocytes.

Effect of metabolic inhibitors.

In these experiments 0·1 ml. of the solution under test was added to 1 ml. of blood. The materials tested were potassium cyanide (10^{-2}M.), mercuric chloride (10^{-3}M.), iodo-acetic acid (10^{-2}M.) (provided by the Department of Biochemistry, University of Sydney), sodium fluoride (10^{-2}M.), and iron ortho-phenanthroline complex (10^{-3}M.) (supplied by Dr. Judith Koch, McMaster Animal Health Laboratories, C.S.I.R.O.). The solutions were all prepared in physiological saline. The results of total uptake and percentage in the haem fraction are shown in Fig. 4.

The experiment with potassium cyanide was performed with reticulocytes induced by phenylhydrazine injections and by haemorrhage. The remaining experiments were performed only with reticulocytes from phenylhydrazine-injected rats. Potassium cyanide in both experiments had no effect on the total uptake, but it reduced significantly the percentage of iron in the haem fraction. On the other hand, mercuric chloride did not affect the proportion of iron in the haem and non-haem fractions, but almost completely inhibited the

uptake of iron. Iodo-acetic acid and sodium fluoride did not affect either process. The iron-orthophenanthroline complex, which is a specific inhibitor of cholinesterase, did not influence the uptake. The results of this experiment are not shown in Fig. 4.

The effect of these metabolic inhibitors on the respiration of reticulocytes was measured by the Warburg respirometer and the results are shown in Table 4.

TABLE 4.

Effect of metabolic inhibitors on the respiration of rat reticulocytes.

Test solution	O_2 consumption (microlitres per hour)	p.c. inhibition	R.B.C. ($\times 10^6$)	Reticulocytes p.c.
KCN (10^{-3} M)	2·70	87	2·88	45·0
$HgCl_2$ (10^{-4} M)	12·45	43	5·99	36·0
Iodoacetic acid (10^{-4} M)	12·80	29	4·56	4·2

The most marked inhibition of respiration was found with potassium cyanide. In rat blood with a low reticulocyte count the inhibition was less marked, suggesting that most of the respiratory activity of blood is due to the reticulocytes. This confirms the previous observation of Warren (1948), who used rabbit blood in his experiments.

Effect of chelating agents.

Ethylene di-amino-tetracetic acid (disodium salt) was used in two ways. Blood containing reticulocytes was incubated with $Fe^{59}Cl_3$, and the cells washed five times with physiological saline, three times with 2 p.c. EDTA, and finally twice with physiological saline. The Fe^{59} content of these cells did not differ from the Fe^{59} content of cells incubated with the same amount of $Fe^{59}Cl_3$, then washed ten times with physiological saline and not with EDTA.

In the second group of experiments, blood containing phenylhydrazine-induced reticulocytes was mixed with 2 p.c. EDTA in physiological saline (1 ml. EDTA to 1 ml. blood). The mixtures were incubated for four hours and then washed six times with physiological saline. The cells were re-suspended in pooled rat plasma and 0·1 ml. of the $Fe^{59}Cl_3$ solution added. After a further period of incubation for two hours the cells were washed 10 times and assayed for iron uptake. The results indicate that EDTA may inhibit the uptake of iron by the reticulocytes but this could not always be demonstrated. The pH of the mixture was shown not to be a factor responsible for the variation.

Similar experiments were performed with α-α'-dipyridyl (11·6 mg. per ml. saline) and with orthophenanthroline (8·4 mg. per ml. saline). One ml. aliquots of rat blood were incubated for four hours with 1 ml. of the solution, and in another experiment with 1 ml. of the solution diluted 1:10. The cells were then

washed six times with physiological saline before the addition of radioactive iron. Both α-α'-dipyridyl and orthophenanthroline inhibited the uptake of Fe^{59} to a marked extent. When the more concentrated solutions were used the counts in the washed cells were usually less than 20 p.c. of those in the controls. A less and somewhat variable inhibition was found with the diluted solutions.

Effect of preliminary loading of cells with iron solution.

Aliquots of rat and of pigeon blood (1 ml.) were incubated at 37° C. for three hours with 0·2 ml. of a solution containing 100 μg. of either ferrous or ferric iron. The cells were then washed three times with physiological saline and re-suspended in 0·9 ml. of rat or pigeon plasma. $Fe^{59}Cl_3$ was added (0·1 ml.); after a further hour hours at 37° C., the cells were washed, haemolysed, and the Fe^{59} assayed. The results seen in Table 5 show that the subsequent uptake of Fe^{59} by both rat and pigeon cells was greatly reduced by prior exposure to iron. There is no difference in this regard between ferrous and ferric iron.

TABLE 5.

Uptake of Fe^{59} by erythrocytes after preliminary exposure to non-isotopic iron.

Experiment	Rat erythrocytes		Pigeon erythrocytes	
	Total counts in sample	p.c. of control	Total counts in sample	p.c. of control
Control	28,735	—	19,378	—
Prior exposure to ferrous iron (100 μg/ml. blood)	6,724	23·4	7,497	39
Prior exposure to ferric iron (100 μg/ml. blood)	6,195	21·6	7,299	38

DISCUSSION.

The rapidity with which iron enters the rat reticulocyte is striking and apparently the iron is used almost immediately for the synthesis of haem. This has been observed by other workers (Jandl, 1959; Belcher and Courtenay, 1959), but our results showed an even more rapid uptake and utilisation than was found by these workers. Jandl observed that the rate of uptake differed according to whether the cells were suspended in saline or in plasma. This was not a feature of our experiments, but a variable total uptake was found when the cells were suspended in saline. This variability was not investigated, but it is possibly the result of changes in the reticulocyte membranes when the cells are suspended in saline during incubation.

The pattern of uptake and haem synthesis in pigeon erythrocytes differed from that in rat reticulocytes. During the first eight hours a linear relationship with time was observed, but after this period the rate of uptake decreased. At

two hours little iron had entered the pigeon erythrocytes, whereas at the same time 80 p.c. of the total had entered the rat cells. At 24 hours the amount of Fe^{59} per unit cell was approximately the same in both rat and pigeon cells. This finding differs from that of Jensen *et al.* (1953) who claimed that duck cells took up about five times as much iron as rat reticulocytes. It is possible that the rate of entry of iron is largely determined by the simultaneous synthesis of acceptors in both pigeon and rat, and that this is more rapid in the rat than in the pigeon. The rate of this synthesis in pigeon cells may be reduced after eight hours because the substrate materials become exhausted *in vitro* or the pH value may change.

Allfrey and Mirsky (1952) were able to show incorporation *in vitro* of N^{15}-labelled glycine into the haemoglobin of avian erythrocytes. Jensen *et al.* (1953) calculated from their experiments with duck erythrocytes that the amount of free protoporphyrin would have to be increased 6- to 20-fold for the formation of all the Fe^{59}-tagged haemoglobin resulting from the addition *in vitro* of Fe^{59} to blood samples. Apparently the avian cell remains nucleated throughout its lifetime in the circulation, and synthesis of haemoglobin precursors may occur throughout this period. Even if haemoglobin synthesis is not continuous (approximately 20 p.c. of the cells in our samples contained a reticulum), it must be a prolonged process.

Mammalian reticulocytes are also able to synthesise haemoglobin *in vitro* from Fe^{59} and labelled glycine (London, Shemin and Rittenberg, 1950), and the amount of protoporphyrin required for the Fe^{59}-haemoglobin in the experiments described is of the same order as in the avian cells. The process is, however, time-limited and probably does not exceed the 36 hours necessary for loss of reticular material as observed by Heath and Daland (1930). During this time the synthesis must be more rapid than in avian cells. It is significant that the rate of respiration of mammalian reticulocytes has been found to be two to four times that of avian erythrocytes (Warren, 1948; quoted by Ponder).

With the human samples the pattern of uptake and the amount entering the reticulocyte was variable. Belcher and Courtenay (1959) found that the rate of iron uptake by reticulocytes is correlated with their number and age distribution. It is well-known that the amount of reticular material in reticulocytes varies considerably, but no detailed morphological study has been made of this aspect in relation to the rate of iron uptake. It is also possible that the disease condition producing reticulocytosis in humans may play some part in the rate of uptake, and it is proposed to investigate this aspect in the future.

The changes in the amount both of iron uptake and haem synthesis as a result of preliminary incubation of the blood samples are of interest. There are two possible explanations of the observations. The first is that incubation results in maturation of the reticulocytes, so that the amount of iron which can enter the cell is limited. The nature of this maturation, which must obviously occur at some stage in the development of mature erythrocytes from reticulocytes,

is not known. The second possibility is that the substrate materials become depleted, or the pH changes, and metabolic processes providing energy for the uptake of iron diminish in activity. Pigeon erythrocytes differed from rat reticulocytes in their behaviour on incubation. A reduction in iron uptake by pigeon cells occurred only after long incubation, whereas the reduction with rat reticulocytes was rapid and marked. This is possibly related to a different metabolic pattern and does not conflict with the suggestion made earlier, that the pigeon erythrocytes may be synthesising iron acceptors for a longer period than rat reticulocytes.

The results with metabolic inhibitors are consistent with the hypothesis that there are two phases in the incorporation of Fe^{59} into haem. Mercuric chloride inhibits the uptake of iron, but does not alter the synthesis of haem. This may be due to an inhibition of sulphydryl-containing enzymes or to non-specific precipitation of proteins which may alter the reticulocyte envelope. By contrast, potassium cyanide, which inhibits oxidative respiration by specific inhibition of copper, zinc and iron-containing enzymes, does not prevent the entry of iron but inhibits the synthesis of haem. The incorporation of iron into the protoporphyrin molecule is therefore an energy-requiring process; on the other hand, inhibition of DPN and calcium- and magnesium-containing enzymes does not impair either phase.

Preliminary incubation of both rat and pigeon blood cells with non-isotopic iron significantly reduces the subsequent uptake of Fe^{59}; this is not surprising in view of the rapidity of haem synthesis. The similarity of the results with ferrous and ferric iron means that the valency of iron is not important in the mechanism of entry. The action of the chelating agents cannot be explained, and it is not known whether they act by chelating metallic ions or by some non-specific action. In the experiments described they must have acted either on the cell surface or within the cell, because the cells were washed six times with physiological saline before the addition of $Fe^{59}Cl_3$. It may be significant that the chelating agents which were most active in reducing iron uptake were those with the greatest affinity for ferrous iron. No evidence was obtained that they inhibited the synthesis of haem, and the effect was limited to the passage of iron into the cell.

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RELEASE OF HISTAMINE FROM MAST CELLS BY ANTIGEN-ANTIBODY REACTIONS *IN VITRO*

by G. T. ARCHER

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

(*Accepted for publication 6th January, 1960.*)

SUMMARY.

The mechanism of mast cell disruption and release of histamine following antigen-antibody reactions was investigated by means of an *in vitro* system using mast cells isolated from the rat. Following the reaction between antigen and fresh immune rat serum, a substance was produced which caused the disruption of added mast cells with the release of histamine. This substance was labile at 37° C., becoming inactive within 10 minutes. Its formation depended on the presence of complement as well as antigen and antibody.

INTRODUCTION.

It is known that histamine may be released when antigen is injected into an animal which has been immunized against the antigen. Recent work has shown that the histamine is released from tissue mast cells, and histological studies have revealed disruption of mast cells coincident with its release (Riley, 1959; Humphrey and Mota, 1959). However, the mechanism of the mast cell damage following antigen-antibody reactions is still obscure. This problem has been investigated with an *in vitro* system in which antigen was added to fresh immune serum. At 37° C. and at pH 7 a labile substance appeared in the serum which caused disruption of added mast cells with release of histamine.

MATERIALS AND METHODS.

Mast Cells.

Mast cells were obtained from the peritoneal cavity of the rat by the method described previously (Archer, 1959a). The mast cells were not separated from other peritoneal cavity cells except in one experiment performed to confirm that presence of the other cells was not necessary for mast cell disruption.

Histamine.

Histamine was estimated chemically by the method of Lowry, Graham, Harris, Priebat, Marks and Bregman (1954).

Immunization of animals.

Human red cells were thrice washed in saline and made up to a 25 p.c. suspension in physiological saline solution. One ml. of the suspension was injected into the thigh of the rat on two occasions a week apart. Blood was obtained more than five days after the last injection.

Human gamma-globulin prepared by the Cohn ethanol fractionation process was supplied as a 16 p.c. solution by the Commonwealth Serum Laboratories, Melbourne. The protein was precipitated with aluminium potassium sulphate by the method of Proom as described by Dacie (1954). 0·5 ml. of this suspension was injected into the thigh of the rat weekly for 12 weeks. Blood was obtained five days after the last injection.

Bovine albumin prepared by the Cohn process and supplied in powder form by the Commonwealth Serum Laboratories was made up to a strength of 1 p.c. in physiological saline solution. A volume of this solution was emulsified with an equal volume of Freund's adjuvant. A total volume of 1 ml. of this emulsion was injected into the subcutaneous tissue of the back and hind legs at weekly intervals for six weeks. Blood was collected more than five days after the last injection.

Collection of blood samples.

Blood was obtained from the jugular vein of Wistar strain albino rats. A small incision was made over the jugular vein prior to venepuncture. The blood was collected with a dry syringe and needle, transferred to a centrifuge tube and covered with a layer of liquid paraffin. Sera were left under paraffin throughout all experiments in order to prevent a rise in pH. Heat-inactivated serum was prepared by heating fresh serum at 56° C. for 30 minutes. Hydrazine-inactivated serum was prepared by a method similar to that described by Taylor and Leon (1959). Two drops of 0·1 M hydrazine solution at pH 7 were added to eight drops of serum and the mixture incubated at 37° C. for 45 minutes.

Antigens for in vitro *test.*

A 25 p.c. suspension of thrice-washed human red cells was prepared in physiological saline solution. When required the cells were lysed by freezing and thawing. Human gamma-globulin and bovine albumin solutions of the required strength were prepared in physiological saline solution.

In vitro *system.*

A mixture consisting of five drops of fresh immune serum, one drop of the mast cell suspension and one drop of the antigen solution was prepared in a test tube and covered with liquid paraffin. The tube was placed immediately in a water bath at 37° C. After three minutes, one drop of the mixture was placed on a slide and examined under the microscope for mast cell disruption. Lysed red cells were added, instead of intact red cells, in experiments with fresh normal serum, heat-inactivated immune serum and hydrazine-inactivated immune serum because, in the absence of haemolysins, it was difficult to observe mast cells in preparations containing intact red cells.

In some experiments with human red cells the reaction mixture was held at 0° C. for 30 minutes. At this temperature the red cells were agglutinated but did not lyse until the mixture was warmed at 37° C. Red cells were also sensitized at 0° C. for periods up to 60 minutes in the absence of mast cells. The sensitized red cells were separated from the immune serum by centrifugation at 0° C. and washed once in buffered saline (pH 7) at 0° C. One drop of the washed sensitized red cells was added to five drops of fresh normal rat serum in the presence of one drop of mast cell suspension. After incubation at 37° C. for three minutes the mixture was examined for haemolysis and mast cell disruption.

RESULTS.

Experiments with human red cells as antigen.

The following observations were made:

The mast cells were seen to disrupt and release histamine, as determined chemically, two or three minutes after antigen, fresh antiserum, and mast cells

were mixed together at 37° C. and at pH 7. The red cells had haemolysed within this period.

In other experiments mast cells were added at various times after mixing the antigen and antiserum, and were examined immediately after addition. It was found that disruption occurred when the cells were added after three minutes and up to 10 minutes, but they remained intact if added after 10 minutes.

When a mixture of lysed red cells, fresh antiserum and mast cells was made at 0° C. the mast cells remained intact. When a similar mixture with intact red cells was heated to 37° C. haemolysis and mast cell disruption occurred. A mixture at 0° C. with intact cells was washed at this temperature with physiological saline and fresh normal rat serum added. Haemolysis and mast cell disruption also took place when this mixture was heated to 37° C. It did not occur if the fresh normal serum was not added.

The rôle of complement in mast cell disruption was investigated by substituting heat-inactivated immune serum and hydrazine-inactivated immune serum for the fresh immune serum in the antigen, antibody and mast cell mixtures at 37° C. No mast cell disruption was observed.

Red cells were sensitized at 0° C. with fresh immune serum. The serum was separated, mast cells were added to the serum, and the mixture was warmed to 37° C. No disruption of mast cells occurred.

Experiments with human gamma-globulin as antigen.

Mast cells were found to disrupt following the addition of antigen to immune serum at 37° C. and pH 7. Activity against mast cells appeared over a range of antigen concentrations from 0·005 p.c. to 0·05 p.c. human gamma-globulin in immune serum. The factor acting on mast cells appeared in the serum within several minutes, and disappeared within 10 minutes of the addition of the antigen. Mast cells did not disrupt following the addition of antigen to fresh normal serum or to heat-inactivated immune serum. The precipitate which formed when antigen and fresh antiserum were mixed was washed with physiological saline solution. The washed precipitate did not affect added mast cells.

Experiment with bovine albumin as antigen.

Mast cells disrupted and a precipitate formed when antigen was added to fresh immune serum at 37° C. and pH 7. The final concentration of the antigen in the mixture was 0·01 p.c. Activity against mast cells remained in the mixture for only a short period after the addition of antigen. Mast cells did not disrupt when bovine albumin was added to fresh normal serum or to heat-inactivated immune serum.

DISCUSSION.

The work reported shows that a labile substance is formed, capable of disrupting mast cells, when antigen is added to fresh immune serum *in vitro* at

37° C. and pH 7. The substance was not formed if the immune serum was heated or treated with hydrazine to inactivate complement. It was also shown that if the reaction was carried out at 0° C. the labile substance was not formed at this temperature but appeared when the temperature was raised to 37° C.

In the reaction between antigen, antibody and complement the sequence of events in the formation of the labile substance which disrupts mast cells can be summarised as follows:

Antigen Antibody
+
Complement
↓
Labile Substance
↓
Disruption of Mast Cells
↓
Liberation of Histamine

Levine, Mayer and Rapp (1954) state that, although haemolysis of sensitized red cells does not occur at 0° C., C′1, C′2 and C′4 components of complement are fixed to the red cells at this temperature. When the temperature is raised to 37° C. the C′3 component of complement is fixed (Rapp, Sims and Borsos, 1959) and haemolysis of the red cells occurs. It is probable that the components of complement are fixed in this sequence at 37° C. in antigen-antibody reactions of the type described in this paper. Since the labile substance disrupting mast cells is not formed at 0° C. (when the C′1, C′2 and C′4 components are presumably fixed) but is formed at 37° C., it seems probable that its formation is consequent upon the fixation of the C′3 component.

The nature of the labile substance is at present unknown, as is the reason for its rapid disappearance. It is not known whether a similar mechanism operates *in vivo*. This work suggests that the labile substance may be the factor, or one of the factors, which liberates histamine from mast cells in animals following some antigen-antibody interactions. However, attempts to demonstrate a similar substance in the serum of animals in anaphylaxis were not successful. This failure may be due to the lability of the substance.

Osler, Randall, Hill and Ovary (1959) showed that when an immune aggregate (consisting of the washed precipitate resulting from the addition of egg-white to anti-egg-white serum) was incubated with fresh rat serum, a substance was formed which caused contractions of guinea-pig ileum in a Schultz-Dale bath. This substance they called "anaphylotoxin", and the strength of the smooth muscle contractions was correlated with the amount of the C′3 component of complement that was fixed. At the moment the relation of anaphylotoxin to the labile substance described in this paper is not clear, since anaphylotoxin is presumably stable.

It has been shown (Archer, 1959b) that when dextran or zymosan is added to normal rat serum, a stable substance is formed which disrupts mast cells and releases histamine. Osler *et al.* reported that when some samples of dextran were added to fresh rat serum a substance acting on guinea-pig ileum was formed with fixation of C'3. It would seem that, in the dextran-serum reactions, the stable mast cell disrupting substance reported by Archer may be similar to the substance reported by Osler *et al.*

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THE GROWTH OF THREE STRAINS OF INFECTIOUS LARYNGOTRACHEITIS VIRUS OF FOWLS IN TISSUE CULTURE

by M. F. PULSFORD

(From the Institute of Medical and Veterinary Science, Adelaide).

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SUMMARY.

The growth in tissue culture of a virulent epidemic type of I.L.T. virus, fully adapted to tissue culture is described. It has not been possible to demonstrate a one-step type of growth, but it is apparent that the eclipse phase of this virus is relatively long, of the order of 18 hours, and is followed by rapid production and release of virus.

A prolonged lag phase is noted in the one strain of low virulence examined. This is in keeping with the growth of such strains, as described by other workers.

INTRODUCTION.

The virus of infectious laryngotracheitis (I.L.T.) was first grown in tissue culture by Beach (1932), who carried it through a number of cultures but reported no details. Atherton and Anderson (1957) carried out a number of serial passages with virus strain H2184 in cultures of chick embryo cells and demonstrated clearly that although it multiplied readily within the cells it was not released into the medium. They showed that in their cultures the virus spread from cell to cell in the monolayer. They described the cell damage in some detail and recorded a growth curve of the virus.

The aim of the present work was to study the growth cycle in more detail and to determine the effect of continued passage on virulence of the virus, both for eggs and fowls.

MATERIALS AND METHODS.

Eggs.

Fertile hen eggs for tissue culture and for titration of virus on the chorio-allantoic membrane (C.A.M.) were obtained from a farm where the birds are regularly vaccinated against I.L.T. For about two months following vaccination of the young stock, titration results were sufficiently irregular to interrupt the work, but, apart from this, no untoward results appeared in the growth of virus in tissue culture or in the titrations. The eggs were incubated before use in a forced draft incubator at 100° F.

Eggs for the passage and titration of virus on the C.A.M. were prepared by a modification of the method of Nadeje, Tamm and Overman (1955). After the membrane had been

lowered in the manner directed, a triangle of shell was cut and removed as in the method of Beveridge and Burnet (1946). This gave, in our hands, the freedom from non-specific lesions claimed for the former method and the ready accessibility of the latter.

Tissue cultures.

The tissue cultures described were all prepared from chick embryo lung. Both lungs of a 13-day chicken embryo were removed aseptically and placed in 5 ml. of warmed trypsin solution and incubated for 10 minutes at 37° C. The flasks were shaken vigorously every few minutes until the lungs had broken down into fragments just visible to the naked eye. The suspension was centrifuged at 1,000 r.p.m. for five minutes and the deposit resuspended in 1 ml. of medium. Because of variation in the size of the tissue fragments, no attempt was made to count the cells, the whole lung being regarded as a unit and the whole yield from five embryos distributed in 12 flasks.

Cultures were set up in Carrel flasks 2-in. in diameter, sealed with rubber stoppers. Flasks each contained 3 ml. of medium consisting of three parts of Hanks (1949) solution to one part of horse serum, together with 100 units of penicillin and 200 micrograms of streptomycin per millilitre.

Except for special purposes, the medium was not changed throughout the life of a culture, although sufficient acid was developed to turn the phenol red in the medium completely yellow after about two days. No attempt was made to control the pH by gassing or removal of stoppers, preliminary experiments having shown insignificant differences between cultures with varying pH and those in which the pH was carefully controlled.

Cultures were inoculated with either 0·1 ml. of suspension of a ground infected C.A.M. in 2 ml. of broth or with 0·3 ml. of medium from a previous 2- or 3-day tissue culture. The virus was inoculated either at the time of first setting up the culture or after incubation for three days to allow the formation of a monolayer. In serial passage and in some early growth curve experiments the inoculum remained in contact with the culture throughout its life. In the detailed growth curve experiments, the inoculum was washed out by changing the medium four times in rapid succession.

All incubation of eggs and cultures was at 37° C. Glassware and rubber stoppers were cleaned as described by Parker (1950).

Virus strains.

Three virus strains were used. The first two, 213/25 from a batch of Commonwealth Serum Laboratories vaccine of that number, and "W" from tracheal exudate received from Arthur Webster Pty. Ltd., were both highly virulent when received as both had been passed regularly in birds to maintain virulence. The third strain, 353/55, a low virulence strain, was isolated from an outbreak of a chronic respiratory disease in South Australia (Pulsford, Chamberlain and Topham, 1956).

Titration of virus.

The titration of virus in eggs was done by the method of Beveridge and Burnet (1946). Free virus was determined by removing 0·1 ml. of medium from the flask after gentle rocking and titration in serial tenfold dilutions. To determine whether this procedure was detecting virus within cells which had become detached from the glass, a number of titrations were carried out on centrifuged deposit and supernatant of this material. Up to 48 hours very little of the virus in the culture supernatant was bound to cells. After this time many cells became detached from the glass and the figure for "free" virus contains a variable and increasing proportion of virus attached to cells free within the medium.

The cell-bound virus was determined by replacing the medium with 3 ml. of 0·25 p.c. trypsin solution for five minutes. The flask was then vigorously shaken and the suspension centrifuged at 1,000 r.p.m. for five minutes. After removal of the trypsin, the cells were

resuspended in 1 ml. of penicillin, streptomycin broth, frozen once in an acetone dry-ice mixture, thawed and titrated as above. Titrations of the trypsin supernatant failed to show appreciable quantities of virus.

EXPERIMENTAL.

Serial tissue culture passage of strain 213/25.

The tissue culture passage series was initiated by inoculating two freshly set up tissue cultures with 0·1 ml. of a broth suspension of chorio-allantoic membrane infected with strain 213/25. The series was maintained by passing 0·3 ml. of supernatant to new cultures every Monday, Wednesday and Friday. Where it was necessary to interrupt this schedule, the culture was seeded into eggs, the membrane harvested three days later and stored at — 20° C. Culture supernatants were also stored, but were usually found to have lost infectivity to such an extent that the egg material was used to reinitiate the series. The history of this strain, since isolation from the vaccine, is 15 egg passages followed by 44, 11, 20, 18 and 54 tissue culture passages with an interpolation of one egg passage after each series.

Effect of tissue culture passage on pathogenicity for eggs.

The virus maintained in eggs regularly gave rise in 3-4 days to an acute fatal haemorrhagic reaction when inoculated with light scarification into the trachea of susceptible cockerels. This character was modified during tissue culture passage as seen in Table 1. In the first few passages the symptoms were

TABLE 1.

Pathogenicity of tissue culture virus for fowls.

Passage	Dose	Symptoms	Result
Egg passage	19,000 P.F.U.	Acute haemorrhagic tracheitis 3rd day	Died
3rd T.C.P.	40 P.F.U.	Acute haemorrhagic tracheitis 4th day	Moribund destroyed
4th T.C.P.	8,000 P.F.U.	Acute haemorrhagic tracheitis 5th day	Moribund destroyed
13th T.C.P.	2,400 P.F.U.	Mild haemorrhagic tracheitis 5th day	Survived
107th T.C.P.	5,000 P.F.U.	Mild haemorrhagic tracheitis 4th day	Survived

longer in making their appearance. By the thirteenth passage the disease produced was milder and most birds survived. There was little further change up to the 107th passage.

This virus maintained in tissue culture passage was used for a number of growth curve studies.

Growth curve with virus adapted to tissue culture.

Observations in the passage series above showed that most of the virus produced was present in the supernatant medium. In the preliminary studies of the growth curve the supernatant medium of single culture was therefore

sampled periodically and titrated to obtain a general picture of the virus growth. The first of these experiments was to examine the relation of various concentrations of virus in the inoculum to the final yield. The inoculum was prepared by diluting the supernatant medium of a 3-day tissue culture of 81st passage, to give a total of $\log_{10}$ 4·52, 3·34, 2·64, 1·96 and 1·24 egg pock-forming units (P.F.U.) in cultures A to E respectively. Each culture was sampled daily and titrated. The results are shown in Fig. 1. It can be seen, firstly, that a successful culture was initiated by the smallest dose used (log 1·24 or approximately 18 P.F.U.), secondly, that the yield of virus at 48 and 72 hours increased with increasing inoculum up to an inoculum of log 3·34 P.F.U. and that no greater yield was obtained from a further 1·7 logs increase in virus concentration. The cultures all yielded about the same amount of virus by the 4th day irrespective of the size of the inoculum.

The second series of experiments was aimed at producing a one-step growth curve from which measurement could be made of fundamental aspects of the

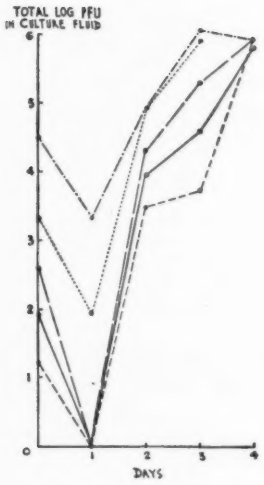

Fig. 1. Daily observations on tissue cultures inoculated with graded dilutions of the medium from the 82nd tissue culture passage of I.L.T. virus strain 213/25.

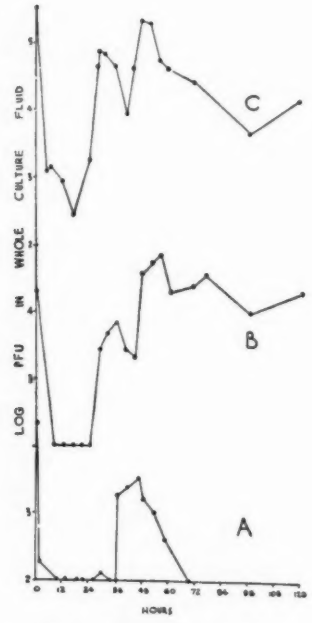

Fig. 2. Growth curves of I.L.T. virus strain 213/25. Observations were made every four hours for the first 60 hours.

growth of this virus. Three curves are illustrated in Fig. 2 (a to c). In these three experiments the inoculum was washed out after allowing two hours for adsorption of the virus to the cells. In the experiment illustrated in Fig. 2a the tissue cultures were set up and the virus added at once. When the cultures were washed two hours later most of the cells were lost as well as the unadsorbed virus inoculum. This curve is included to show that the effects produced are not associated with maturation of the cell sheets in the cultures. In both Figs. 2a and b the inoculum consisted of 0·3 ml. of the supernatant medium from the previous tissue culture. In Fig. 2b and all succeeding growth curve experiments the cultures were incubated for three days before inoculation to allow the formation of sheets. The inoculum obtained from the tissue cultures appeared to be too dilute to infect all susceptible cells as judged from the apparent occurrence of a second generation of virus at 48 hours. Higher concentrations were therefore used in subsequent cultures by passing the virus once in eggs and inoculating the experimental flasks with 0·1 ml. of a suspension of C.A.M. In the case of Fig. 2c this gave a concentration more than two logs above that shown to be adequate for maximum yield in the earlier experiments.

In all cases, the curve was similar in shape; soon after 24 hours there was a rapid release of virus, and immediately before 48 hours there was a second release, giving a second peak about one log higher than the first. The second peak was followed by a tail maintaining a relatively high virus content in the culture for some days.

The final experiment in this series was designed to examine the early appearance of virus in greater detail. The cultures were set up as for Fig. 2c above, but observations of both free and cell-bound virus were carried out every two hours from 18 hours. The results are shown in Fig. 3. This curve shows a rapid production of virus within the cell soon after 18 hours, with its almost immediate release into the medium. The curve of free virus was constructed from serial samples of a single culture and that for the bound virus from a separate culture at each point. It is therefore not possible to give a direct measure of the rate of release. From other observations with this strain it appears that the cultures used for the "free-virus" estimations in this experiment were in fact developing a little more rapidly than most. It has been usual to find that during the early rise of virus the curve of free virus lags 4-6 hours behind the bound virus curve. This is seen in the experiment shown in Fig. 4a.

Comparison of the growth curves of three strains of virus.

Further growth curve studies were carried out with three strains of virus to determine whether the strain or its adaptation to tissue culture was responsible for the differences between these results and those of Atherton and Anderson (1957) in which no free virus was found in the medium. These experiments are illustrated in Fig. 4 (a to c). In Fig. 4a, a full growth curve with four hourly

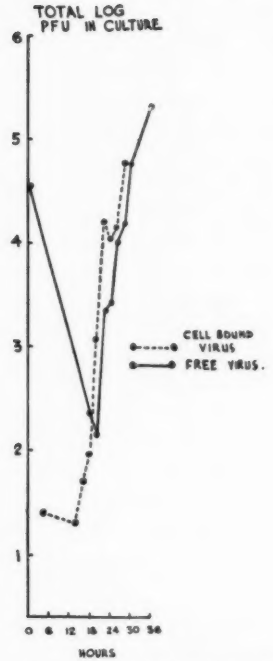

Fig. 3. Growth of I.L.T. virus strain 213/25 in tissue culture following inoculation with 0·1 ml. of 1st egg passage from the 138th tissue culture passage. Observations of bound and free virus were taken every two hours over the period from just preceding the eclipse phase, to the end of the first release of virus.

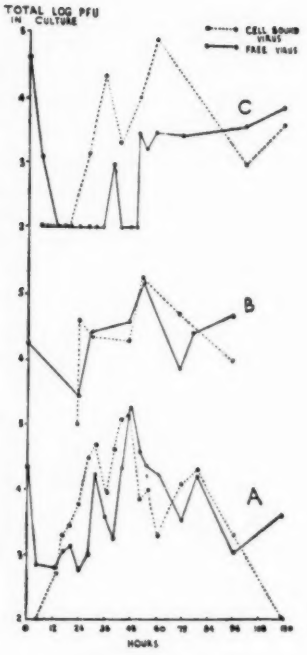

Fig. 4. Showing cell-bound virus and virus free in the medium in tissue cultures of (a) Strain 213/25, (b) Strain W, and (c) Strain 353/55.

observations of both free and bound virus of strain 213/25 at the 119th T.C. passage is shown as a basis for comparison with the other two. Fig. 4b refers to strain W, but with samplings taken only twice daily. These are, however, sufficient to show that much of the virus is liberated into the medium as with the first strain. This strain has not previously been in tissue culture and was used at the second egg passage following isolation from the trachea. In Fig. 4c is shown a complete growth curve of the lowly virulent strain 353/55, but with bound virus measured only every eight hours. It is seen that with this strain virus was liberated slowly into the medium and never in great quantities. In this case the typical double peak is seen only in the curve of bound virus.

Inactivation of the virus in the absence of cells at 37° C. was followed in fresh and exhausted medium. For the fresh medium test the medium was removed from an actively growing culture, centrifuged and 0·3 ml. inoculated into a flask containing 3 ml. of medium but no cells. For the test in exhausted medium the whole medium was removed from a three-day culture, centrifuged and placed in a flask without cells. In each case 0·1 ml. was removed and titrated at intervals as shown. The results are seen in Fig. 5. Inactivation was very rapid in the fresh medium and it can be seen that it could account for the rapid fall of virus content in the period between the first two peaks in Fig. 2. Inactivation in the spent medium was much slower and may account in part for the late tail of the curves as seen in Fig. 2.

Statistical aspects.

Beveridge and Burnet (1946) have shown that in pock counting methods the use of from 4-6 membranes will give results accurate to within ± 50 p.c. of the true mean, with a probability of 95 p.c. They showed that if the mean ($\bar{x}$) is allowed values from $\bar{x}/2$ to $3\bar{x}/2$; $\sqrt{n}$ (when n = the number of membranes counted) must be greater than $4\sigma/\bar{x}$ and writing $\sigma/\bar{x}$ equals C, n must be greater than $16C^2$.

Similarly, it can be shown that if $\bar{x}$ is allowed values from $\bar{x}/3$ to $5\bar{x}/3$, i.e. ± 66 p.c. of the true mean, n must be greater than $9C^2$.

Where the amount of work which can be done is limited by the number of eggs which can be handled and where large differences are being measured, it may be expedient to increase the number of points at which observations are made or even to repeat a whole experiment, rather than increase the number of eggs per sample. In this work three eggs have been used at each dilution level in titrations. Not all experiments have been analysed in detail, but critical points have been checked where necessary. Table 3 shows the value which n (i.e. the number of membranes counted) must exceed for the probability that $\bar{x}$ lies within the limits of 66 p.c. or 50 p.c. of the true mean for each point in Fig. 2c to be 95 p.c. or better. It can be seen that with the three eggs used for each dilution level, the mean probably lies well within the 66 p.c. level except in one case, i.e. 40 hours.

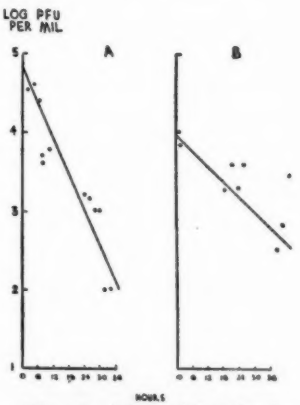

Fig. 5. Thermal inactivation curves of I.L.T. virus strain 213/25 from 103rd tissue culture passage. Curve A shows the rate of decline of titre in fresh tissue culture medium. Curve B shows the decline in virus, when the medium was separated from the cells of a three-day culture.

The above analysis covers only the error involved in the determination of a population in one particular dilution by counting pocks on membranes seeded

TABLE 2.

Showing values which n must exceed for each point in fig. 2b, when the probability is 95 p.c. that the mean of the sample is within certain limits of the true mean.

Permitted variation of mean of sample from true mean	Value of c^2	Hours												
		24	28	32	36	40	44	48	52	56	60	72	97	120
±66 p.c.	9	·36	1·9	1·36	2·58	4·9	1·82	·59	·03	2·7	·57	1·98	·63	2·52
±50 p.c.	16	·64	3·5	2·41	4·59	8·8	3·23	1·06	·05	4·7	1·0	3·52	1·12	4·48
n		2	3	5	3	3	5	3	2	6	3	3	3	5

$c^2 = \frac{\sigma}{\bar{x}}$ n = no. of membranes counted.

from that particular dilution. To cover errors in sampling and dilution, the five points concerned with the step in the graph in Fig. 2c have been examined by the "t" test to determine whether the second three points are significantly lower than the first two. Taking the count of each membrane separately as an estimate of the population at 28 and 30, 32 hours on the one hand and 36, 40, 44 hours on the other, $t = 3 \cdot 724$ and P is less than $0 \cdot 01$.

The significance of this analysis is, firstly, that the step between the two separate rises in free virus content is real, and secondly, that any point on the graphs should be within 75 p.c. of the true mean.

DISCUSSION.

Serial passage in tissue culture of strain 213/25 did not alter the appearance of focal or confluent lesions on the chorio-allantoic membrane, but during the first 13 passages there was some loss of virulence for birds. The prolonged passage in tissue culture does not appear to have greatly affected its growth in this medium. The conditions for this passage series were, however, very different from those of Atherton and Anderson (1957), who were unable to pass the virus without disrupting the cells of the old culture.

It was the original intention of this work to study a one-step growth curve. Despite all efforts to this end, e.g. by increasing the concentration of the virus in the inoculum, agitation during the period of absorption, and decreasing the number of cells in the culture, it was not possible to alter the essential shape of the curve, provided a certain minimum of virus was used. The virus first appeared just before 24 hours and soon reached a peak; a second peak usually about one log higher occurred at about 48 hours followed by a relatively high virus level lasting for some days.

Including the present work, tissue culture growth curve studies have, to our knowledge, been done on five strains of I.L.T. virus. With two of our virulent epidemic type strains, 213/25 and "W", it is seen that any level of cell-bound virus is reached in the free virus curve within six hours. With our third strain, 353/55, which was of the low virulent endemic type, the bound virus levels were not reflected in the free virus curve until 12-24 hours later. The fourth strain was the endemic, lowly virulent type "S" of Burnet (1936) which was tested by Atherton (1958). He showed that virus was released, but as with our endemic type strain, any level of bound virus was not seen in the free virus curve until 18-24 hours later. The fifth strain known to have been examined in this way was isolated in Queensland by Simmons (1958), who states that the virus was of low virulence, being isolated from a chronic respiratory disease in which there were a number of other aetiological agents present. It was tested by Atherton and Anderson, whose critical experiments demonstrate that only very minute quantities of virus are released into the medium, at any time up to about six days.

The significance of this difference between several strains in the release of the virus into the medium is not at present clear. The difference has been recorded both in this laboratory and by Atherton, but the number of strains studied so far is too small for it to be stated that this characteristic is associated either with virulence or with the epidemiological characters of the virus. Of these five, however, the two strains, 213/25 and "W", of epidemic highly-virulent type, released virus from the cells into the medium within six hours of its appearance within the cells. Two strains of an endemic type, one "S" of moderate virulence and the other 353/55 of low virulence, released some of their virus into the medium within 12 to 24 hours. The fifth strain, H2184 of low virulence and endemic type failed to release virus into the medium except in minute quantity over about six days.

Acknowledgments. Our thanks are due to Mr. Arthur Webster, of Arthur Webster Pty. Ltd., Northmead, N.S.W., for the supply of a highly virulent strain of virus. Thanks are also due to Miss Louise Verner for careful technical assistance, especially in the long period of continuous observation.

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GLUCOSE AND NITRATE UTILISATION BY ANAEROBIC CULTURES OF *PS. AERUGINOSA*

by F. M. COLLINS

(From the Department of Microbiology, University of Adelaide, South Australia).

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SUMMARY.

Ps. aeruginosa was grown under anaerobic conditions in a synthetic glucose nitrate medium. In the presence of nitrate, carbon dioxide and cellular carbon were the end products of glucose metabolism. The nitrate was reduced almost entirely to gaseous nitrogen or nitrous oxide (18 p.c. of utilised NO_3-N). Continued incubation of anaerobic cultures after the exhaustion of the nitrate did very little to alter the end products of nitrate utilisation, but striking variations were recorded for the glucose utilisation. Up to 11 p.c. of the utilised glucose carbon was recovered as volatile and non-volatile organic acids. The non-volatile acids included extensive amounts of lactic and glycollic acids together with smaller quantities of succinic, malic, fumaric, citric, α keto glutaric and oxalacetic acids. The volatile acid was produced in approximately the same quantity in both aerated and anaerobic cultures whether nitrate was present or not. Carbon balances of 100 p.c. were obtained for the anaerobic cultures, but extensive discrepancies in the aerated culture indicated the formation of other undetected end products of glucose metabolism.

INTRODUCTION.

The utilisation of organic carbon compounds by actively denitrifying bacterial cultures has not been extensively studied but is generally believed to involve the oxidation of the energy source to CO_2 and water. Sacks and Barker (1952) reported 90 p.c. recovery of a mixture of succinate and glutamate as CO_2 in denitrifying cultures of *Ps. denitrificans*. The nitrate was reduced to gaseous nitrogen with only a few per cent. of the nitrate-nitrogen detected as cellular protoplasm. Similarly, Verhoeven and Goos (1954) examined the anaerobic utilisation of glucose by *Ps. aeruginosa* cultures and reported 70 p.c. recovery as CO_2 with 25-30 p.c. assimilation. Alcohols and organic acid end products were not considered by either group of workers to be important, because CO_2 and cellular protoplasm accounted for virtually all of the utilised glucose-C. Nitrogen balance sheets showed that gaseous nitrogen and assimilation accounted for most of the utilised nitrate but significant amounts of N_2O were also produced (Verhoeven, 1950; Kluyver and Verhoeven, 1954).

In the present study, certain discrepancies in the carbon but not the nitrogen balance sheets of some anaerobically incubated *Ps. aeruginosa* cultures were

noted. Further investigation disclosed the presence of various organic acids in the culture medium. The presence of these acids appeared to depend largely on the environmental conditions.

Materials and Methods.

Organism. *Ps. aeruginosa* N.C.T.C. 6750 was grown on a digest agar slope for 18 hours at 37° C. A little growth removed from the slope was suspended in 10 ml. of sterile tap water. The suspension was aseptically inoculated into a synthetic liquid medium to supply an initial population of about 10^5 cells per ml.

Medium. This consisted of: glucose, 10·0 gm.; sodium nitrate, 5·0 gm.; potassium dihydrogen phosphate, 80 mg.; disodium hydrogen phosphate, 400 mg.; magnesium sulphate, 50 mg.; manganese sulphate, 2·5 mg.; sodium chloride, 2·5 mg.; ferrous sulphate, 1·0 mg.; glass distilled water, 1 litre. The medium had a pH of 7·0 and was sterilised by autoclaving at 115° C. for 15 minutes.

The medium was dispensed in a 6in. x 2in. Pyrex test tube filled to within an inch of the top. The tube was sealed with a rubber bung fitted with two capillary tubes, one of which led to an inverted measuring cylinder (F) filled with mercury (see Fig. 1). The other tube was closed by a clip at B, and the open end of the tube was protected by a cotton wool plug. The tube was inoculated by drawing up a suitable volume of a tap water suspension of *Ps. aeruginosa* into the tube E by applying a negative pressure to the tube C. The test tube (A) was then completely filled with sterile medium by the same method and the clip B was tightly closed. The rubber fittings and joints were sealed with shellac. The tube E was connected to the measuring cylinder and incubation at 37° C. was continued for up to five days. The evolved gas in the cylinder (F) was measured daily and its composition was later determined with a Haldane gas analysis apparatus. The amounts of carbon dioxide, oxygen, hydrogen, methane, nitrous oxide and nitrogen present were calculated in mg.

Carbon dioxide production by an aerated culture of *Ps. aeruginosa* was estimated gravimetrically in a known quantity of normal potassium hydroxide. The water vapour was first removed from the effluent air with an alcohol dry ice bath before it was bubbled through the KOH. The culture flask was aerated with a brisk stream of CO_2-free air introduced by a sintered glass funnel immersed in the vigorously stirred medium.

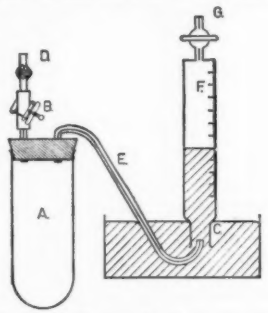

Fig. 1. Gas collection apparatus (see text for the description).

Carbon dioxide dissolved in the medium was estimated in a van Slyke apparatus. The nitrous oxide was first removed by the application of a vacuum to the one ml. sample and the amount present was estimated (Kluyver and Verhoeven, 1954). The CO_2 was later estimated by difference in gas volume after the addition of one ml. of N. sulphuric acid. Total cell counts were carried out by the method of Stähelin (1954) and 10^9 bacteria were found to be equivalent to one mg. wet weight of cells.

On completion of the incubation period the cells were removed by centrifugation, the clear supernatant fluid was heated at 56° C. for 30 minutes and stored at 4° C. until required. Quantitative glucose estimations using the method of Somogyi (1945) were made before and after growth. Lactic acid was estimated colorimetrically by the method given by Umbreit, Burris and Stauffer (1949).

Volatile organic acids in the culture medium were titrated after steam distillation of

the acidified cell-free supernatant fluid. The non-volatile acids were identified chromatographically. Two hundred ml. of cell-free medium were concentrated *in vacuo* to a thick syrup which was then acidified with 10 p.c. sulphuric acid and made up to 10 ml. with distilled water. The concentrate was absorbed onto 7 gm. of acid-washed Hyflo Supercel. The lactic acid was extracted with cold anaesthetic ether and the remaining acids were then removed with freshly distilled ether in a Soxhlet apparatus for three hours. The two extracts were mixed and concentrated to a thick syrup at room temperature in a stream of air. The residue was dried *in vacuo* over concentrated sulphuric acid for 24 hours and finally was dissolved in 2 ml. of 10 p.c. butanol in chloroform (Nossal, 1956).

The acids were separated on ascending paper chromatograms and by column chromatography using the methods reported by Ladd and Nossal (1954). The solvent for the column chromatography was equilibrated with the 0·05 N. sulphuric acid for 24 hours to prevent the elution of mineral acid from the silica gel column. The effluent was cut into fractions by an automatic fraction collector operating on a time basis. One minute fractions were collected for the first 150 minutes, two minute fractions for the next 100 minutes and five minute fractions for the remainder of the experiment. Each fraction was titrated with N/1000 sodium hydroxide in a stream of CO_2-free air.

Total carbon and nitrogen balances were drawn up under the following conditions: (a) anaerobic cultures in which incubation was prolonged for 24 hours after the nitrate supply was exhausted (experiments 1 and 2); (b) nitrate supply exhausted immediately prior to harvesting (experiments 3 and 4); (c) anaerobic cultures containing nitrate (experiment 5); (d) continuously aerated cultures (experiment 6).

EXPERIMENTAL.

Preliminary experiments with anaerobic *Ps. aeruginosa* cultures showed that the absence of nitrate from the medium affected the recovery of the utilised glucose-C (Table 1). The culture containing nitrate gave a recovery of 90 p.c.

TABLE 1.

Carbon recovery in Ps. aeruginosa *cultures grown in a synthetic glucose medium before and after exhaustion of the nitrate.*

	Before nitrate exhaustion	24 hours after nitrate exhaustion
Initial glucose-C (mg.)	1320	1320
Residual glucose-C (mg.)	940	885
Utilised glucose-C (mg.)	380	435
CO_2-C (gaseous and solution mg.)	235	216
Bacterial-C (mg.)	107	98
Carbon accounted for (mg.)	1262	1199
Carbon recovered (mg.)	342	314
p.c. of utilised glucose	90	72·2

of the utilised glucose-C, but if incubation was continued for 24 hours after the nitrate disappeared only 72·2 p.c. was accounted for as CO_2 and cellular carbon. The disparity in the carbon recovery in the nitrate-containing culture was within the limits of experimental error but the 28 p.c. loss observed in the nitrate-free medium was too large to be explained by this means and so other possible end products of the glucose metabolism were sought. *Ps. aeruginosa* cultures do not

produce acetyl-methyl carbinol or ethanol during the utilisation of glucose. However, the formation of acid with no gas had been reported in synthetic glucose-ammonium sulphate medium (French, 1947; Bergey, 1957). Attempts were therefore concentrated on the detection and identification of any organic acids which may have accumulated in the anaerobic culture media.

The C:N ratio of 5:1 in the medium used in the present study was designed to ensure that the nitrate supply was the limiting factor for anaerobic growth.

TABLE 2.

Carbon recovery in Ps. aeruginosa *cultures grown in a synthetic glucose medium.*

	Anaerobic cultures					Aerated
	1	2	3	4	5	6
Initial glucose-C (mg.)	1360	1320	1320	1320	1320	2040
Residual glucose-C (mg.)	873	885	912	900	1135	1770
Utilised glucose-C* (mg.)	487·0	435·0	408·0	420·0	85·0	270·0
Gaseous CO_2-C (mg.)	29·2	41·2	41·7	44·0	16·2	102·8
CO_2-C in solution	158·0	185·0	195·5	191·0	91·4	6·7
Total CO_2-C (mg.)	187·2	226·2	232·2	235·0	107·6	108·5
p.c. of utilised-C	38·4	52·0	58·1	56·0	58·2	40·2
Bacterial-C (mg.)	216·0	198·6	178·2	206·0	52·0	21·5
p.c. of utilised-C	44·4	45·7	43·7	49·0	28·1	8·0
Volatile acid-C (mg.)	15·0	16·4	12·7	6·2	18·0	20·4
p.c. of utilised-C	3·1	3·8	3·1	1·5	8·1	7·6
Non-volatile acid-C (mg.)	40·9	17·0	3·1	2·5	1·4	4·1
p.c. of utilised-C	8·4	3·9	0·8	0·6	0·8	1·5
Total organic acid-C (mg.)	55·9	33·4	15·8	8·7	16·4	24·4
p.c. of utilised-C	11·5	7·7	3·9	2·1	8·9	9·0
Carbon recovered (mg.)	459·1	488·2	431·2	449·7	176·0	154·4
p.c. of utilised-C	94·3	105·3	105·7	107·0	95·1	57·1
Total carbon accounted for (mg.)	1332·1	1393·3	1343·2	1349·7	1311·0	1924·4
p.c. of total	97·9	101·8	101·8	102·3	99·3	94·3

* by difference.

TABLE 3.

Composition of gas evolved during anaerobic growth of Ps. aeruginosa *in a synthetic medium.*

Experiment number	Sample number	Percent of gas					
		CO_2	O_2	CH4	H_2	N_2O	N_2*
1	1	22·3	0·1	0·0	0·0	3·0	74·6
2	1	24·6	0·0	0·0	0·0	4·5	67·5
	2	39·0	0·0	0·0	0·3	4·4	54·3
3	1	23·7	0·8	0·0	0·0	3·6	71·9
	2	32·0	0·9	0·0	0·0	3·0	64·9
4	1	21·7	0·0	0·0	0·0	0·0	77·6
	2	31·5	0·1	0·0	0·0	0·0	67·1
5	1	23·1	0·1	0·0	0·0	3·4	73·4

* estimated by difference.

The glucose supply was, in fact, almost twice that required for the complete growth of the organism under aerobic or anaerobic conditions.

Detailed carbon balances for the *Ps. aeruginosa* cultures grown under the four specified sets of conditions in experiments 1 to 6 are recorded in Table 2. Carbon dioxide production and direct assimilation of the glucose-C accounted for 80 to 100 p.c. of the utilised glucose-C in the anaerobic cultures (Table 2). The yield of cellular carbon was less in the aerated culture than was recorded for the anaerobic ones largely because of the shorter incubation period for this experiment. The pH of the aerated medium quickly fell to 4·0 resulting in the retention of only very small amounts of CO_2 in the medium itself and most of the carbon in this fraction (101·8 mg. or 90 p.c.) was recovered in the KOH trap. The release of sodium hydroxide following the destruction of the nitrate radicle in the anaerobic cultures trapped most of the CO_2 as bicarbonate in the medium. When the nitrate supply was exhausted less CO_2 was retained in the medium and so the later gas sample contained a higher proportion of CO_2, i.e. the first 100 ml. of gas evolved by the anaerobic cultures contained 22-25 p.c. CO_2, but the subsequent sample gave figures of 30-40 p.c. (Table 3). The total amount of CO_2 produced represented between 40 p.c. and 50 p.c. of the utilised glucose-C (Table 2).

The presence of volatile organic acids was shown to be a constant feature of the glucose metabolism of *Ps. aeruginosa*, irrespective of the conditions of incubation (Table 2). The aerated culture produced 20 mg. of volatile acid-C. In terms of acid produced per mg. of glucose-C utilised the aerated culture produced almost twice as much acid in this fraction as the anaerobic counterpart but this still only accounted for 7·6 p.c. of the utilised glucose-C. The volatile acid fraction averaged 13 mg. carbon for the anaerobic cultures 1-5 which corresponded to about 4 p.c. of the utilised glucose-C.

The non-volatile acid fraction showed considerable variation in the type and amount of the various acids present in the medium. The most common acids detected were lactic, glycollic, fumaric, malic, citric and succinic acids while α keto glutaric and oxalacetic acids were also present in certain cultures (Table 4). The carbon figures for this fraction shown in Table 2 revealed a definite trend which appeared to depend on the environmental conditions prior to the harvesting of the culture. The aerated culture (experiment 6) accumulated very little carbon (4·06 mg.) in this fraction. The small amount of acid present consisted mainly of lactic and glycollic acids together with traces of fumaric, malic and citric acids (Figs. 5a and 5b). The quantity of carbon involved (1·5 p.c. of the utilised glucose-C) was too small for the organic acids to be considered significant end products of aerobic glucose metabolism by *Ps. aeruginosa*. Similar results were recorded for those anaerobic cultures in which nitrate was continuously present or had been removed just prior to the completion of the experiment (experiments 3 to 5 in Table 4). Where nitrate was still present,

less than 0·8 p.c. of the utilised glucose-C was detected as lactic, glycollic, malic and citric acid (Figs. 3a, 3b, 4a and 4b) and was therefore ignored as an important growth product. Exhaustion of the nitrate supply had little effect on this pattern unless incubation was continued for an extended period. In the continued absence of an external hydrogen acceptor a striking difference was observed from the above results. In experiments 1 and 2 extensive amounts of lactic, fumaric, citric and glycollic acids were observed (Table 4) together with traces of malic, α keto glutaric and oxalacetic acids (Figs. 2a and 2b).

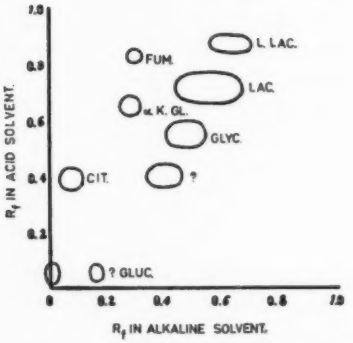

Fig. 2a. Two dimensional paper chromatogram of the ether extract of the medium in experiment 2. L. lac: lactyl lactic acid produced from the lactic acid during the extraction process. Lac: Lactic acid; Fum: Fumaric acid; α k Gl: α keto glutaric acid; Glyc: Glycollic acid; Cit: Citric acid; Gluc: Gluconic acid; ?: unknown acid.

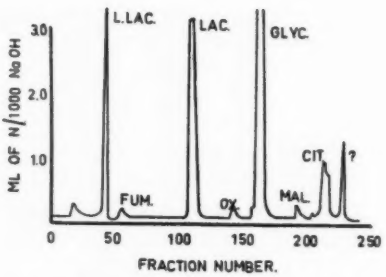

Fig. 2b. Column chromatogram of the ether extract of the culture medium in experiment 2.

In addition, several unknown spots were detected on the paper chromatograms but no definite peaks could be detected in the column chromatogram which could correspond to these unknown acids. The Rf values for the unknown spots did not agree with those for gluconic acid but they may have represented substituted gluconic acid derivatives. Gluconic acid was not eluted from the silica gel column and quantitative estimates of any gluconic acid or its derivatives was therefore not possible. The non-volatile acids accounted for 4-8 p.c. of the utilised glucose-C in experiments 1 and 2 (Table 2). When added to the volatile acid-C these two fractions accounted for 8-11·5 p.c. of the utilised glucose-C and were thus considered as significant end products of glucose metabolism under these particular cultural conditions. When added to the CO_2-C and bacterial-C this organic acid fraction results in carbon recoveries of 94-107

TABLE 4.

Organic acids produced by cultures of Ps. aeruginosa *grown in a synthetic medium.*

Experiment number	Volatile acid-C (mg.)	mg. non-volatile acid-C								
		Fumaric acid-C	Lactic acid-C	Succinic acid-C*	α keto glutaric-C	Oxalacetic acid-C	Glycollic acid-C	Malic acid-C	Citric acid-C	Total acid-C
1	15·0	3·86	34·67	0·02	0·35	0·00	2·02	0·00	0·00	55·9
2	16·4	1·18	11·02	0·00	0·01	0·07	14·8	0·42	2·78	33·4
3	12·7	0·07	2·00	0·06	0·00	0·00	1·01	0·00	0·00	15·8
4	6·2	0·00	0·56	0·02	0·00	0·00	1·60	0·00	0·32	8·70
5	15·0	0·06	0·00	0·00	0·00	0·00	0·86	0·18	0·34	16·4
6	20·4	0·34	1·20	0·03	0·00	0·05	2·06	0·12	0·26	24·4

* this estimate was inaccurate due to the close proximity of the lactic acid peak which rendered difficult the detection of the smaller quantities of succinic acid.

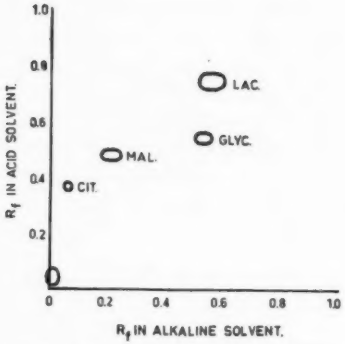

Fig. 3a. Two dimensional paper chromatogram of the ether extract of the medium in experiment 4. Mal: Malic acid.

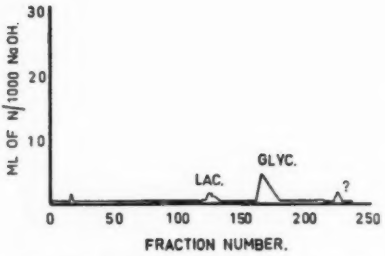

Fig. 3b. Column chromatogram of the ether extract of the culture medium in experiment 4.

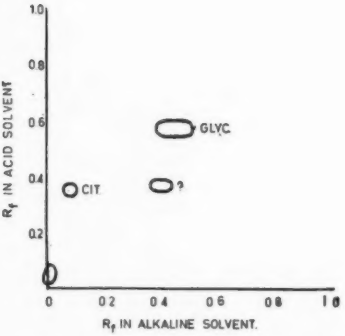

Fig. 4a. Two dimensional paper chromatogram of the ether extract of the medium in experiment 5.

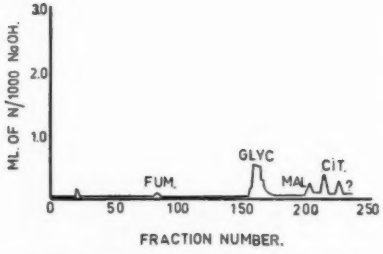

Fig. 4b. Column chromatogram of the ether extract of the culture medium in experiment 5.

p.c. for the five anaerobic cultures (Table 2). The carbon recovery for the aerated culture was not as good, probably due to the accumulation of extensive amounts of the utilised glucose as gluconic acid derivatives or possibly alcohols.

The nitrogen in the gas samples was assumed to be the inert gas remaining after the estimation of the CO_2, oxygen, hydrogen, methane and nitrous oxide in the gas sample. Such an assumption was open to criticism but some check on its validity could be made from the nitrogen balances for these cultures. The recoveries of 98-110 p.c. of the nitrate-N (Table 5) obtained for the anaerobic cultures indicated that this assumption was not unwarranted since deviations from the ideal were within experimental error. Thus the gas analysis was ap-

parently satisfactory and the assumption that all the remaining inert gas was nitrogen appeared to be valid. Only about 20-25 p.c. of the nitrous oxide produced was detected in the gas evolved by the anaerobic cultures, while the remainder was present in the medium. Up to 55·2 mg. N_2O-N was detected in the anaerobic cultures and constituted between 20 and 30 p.c. of the nitrate-N (Table 5). Continued anaerobic incubation in the absence of nitrate did not

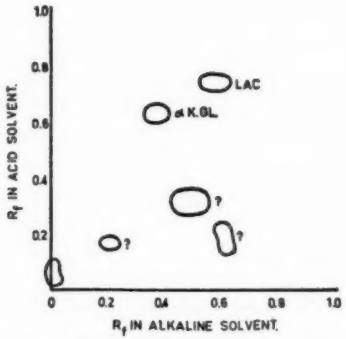

Fig. 5a. Two dimensional paper chromatogram of the ether extract of the medium in experiment 6.

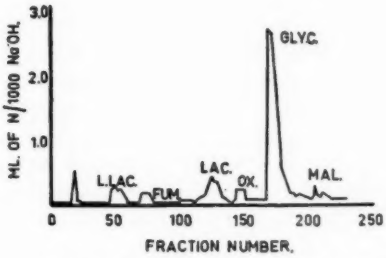

Fig. 5b. Column chromatogram of the ether extract of the culture medium in experiment 6.

TABLE 5.

The nitrogen balance for cultures of Ps. aeruginosa *in a synthetic medium.*

	Anaerobic cultures			Aerated
	1	4	5	6
Initial nitrate-N (mg.)	286·0	280·0	280·0	441·0
Residual nitrate-N (mg.)	0·0	0·0	95·4	284·0
p.c. utilisation	100	100	66·0	35·6
Nitrite-N (mg.)	0·0	0·0	1·0	2·3
Ammonia-N (mg.)	0·0	0·0	15·8	28·6
p.c. utilisation	0·0	0·0	8·6	18·2
Yeast extract-N (mg.)	4·3	4·3	4·3	4·3
Gaseous-N_2 (mg.)	228·5	256·0	118·0	—
p.c. utilisation	80·3	90·0	63·9	—
Gaseous N_2O-N (mg.)	9·2	0·0	5·5	—
N_2O-N in solution (mg.)	46·0	19·6	28·6	27·1
Total N_2O-N (mg.)	55·2	19·6	34·1	27·1
p.c. utilisation	19·3	7·0	18·5	17·3
Bacterial-N (mg.)	22·0	21·2	10·4	19·8
p.c. utilisation	7·1	7·6	5·6	12·6
Total N recovered (mg.)	305·7	296·8	274·7	361·8
p.c. of total-N recovered	106·8	106·0	98·1	82·0

— =not done.

result in the removal of significant quantities of N_2O either from the medium or the evolved gas, at least within the time limit of the present experiments. The nitrogen balance sheets also showed that, as expected, there were no variations in the nitrogenous end products under the varying conditions of the present experiments. The unbalance of the aerated culture clearly demonstrated that aeration was insufficient to inhibit completely all denitrification by the culture.

DISCUSSION.

The principal end products of glucose metabolism by *Ps. aeruginosa* cultures were carbon dioxide and cell protoplasm as was reported by earlier workers. However, the discrepancies in the carbon recoveries of certain anaerobic cultures in the present series of experiments strongly suggested the occurrence of other end products besides CO_2. Examination of the culture media disclosed the presence of varying amounts of organic acids under all the conditions tested. Acetic acid was probably produced in extensive amounts during the growth of the organism, but in the presence of an external hydrogen acceptor the acid was utilised further and did not accumulate in the medium in significant quantities. On the other hand, formic acid was unable to serve as a source of carbon and energy, nor was it oxidised manometrically in the presence of oxygen or nitrate (Collins, unpublished experiments). The organism had no hydrogenlyase activity and so any formate produced would tend to accumulate in the medium. In the absence of an external hydrogen acceptor (expts. 1 and 2) small amounts of acetic acid may have accumulated in the medium but the amount of carbon in this volatile fraction was calculated from the acidity on the assumption that only formate was present. The actual error in the carbon estimation due to this assumption must have been very small and was disregarded.

Lactic and glycollic acids were detected in most of the cultures together with a number of other acids present as traces only. The actual amount of carbon accounted for was much the same for all the experiments except where incubation was continued in the absence of nitrate. Thus prolonged anaerobic incubation affected the normal glucose metabolism of the organism so that certain steps in the oxidative pathway were blocked. This led to the progressive accumulation of intermediaries which could only be utilised further by an oxidative step. Some confirmation for this theory was found in the ability of *Ps. aeruginosa* to grow when lactate, succinate, fumarate or citrate was supplied to the organism as the sole carbon and energy source provided that oxygen or nitrate was also present. No growth occurred in the absence of these hydrogen acceptors. The organism therefore contained the necessary enzymes to utilise the acids completely and only the absence of a suitable hydrogen acceptor led to their accumulation in the medium.

Prolonged incubation of dense suspensions of *Ps. aeruginosa* under strictly

anaerobic conditions in the presence of a suitable carbon source may thus be an easy method for trapping certain intermediates in the glucose utilisation pathway. Normally these intermediates will be present in the medium in undetectable trace quantities, but by appropriate manipulations they may be readily isolated. The present study shows that under certain conditions up to 11 p.c. of the utilised glucose-C may be transformed into organic acids by *Ps. aeruginosa*. The discrepancy may be less than the experimental error and so attempts to detect the presence of these acids must be made before a carbon balance for a denitrifying culture can be completed, irrespective of the agreement between the utilised and recovered carbon figures. On the other hand, the present results for the nitrogen balance agree closely with those of Verhoeven and Goos (1954) and showed no variations irrespective of the cultural conditions employed.

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DIFFERENCES IN ASSOCIATION OF CORTICOSTEROIDS WITH SERUM PROTEINS OF DIFFERENT ANIMAL TYPES[1]

by MAGDA REICH[2]

(From the Department of Physiology, University of Melbourne).

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SUMMARY.

A marked species difference in the capacity of plasma protein fractions to combine with corticosteroids has been demonstrated by the use of C^{14}-labelled cortisol and corticosterone, and paper electrophoresis methods. Of the five species studied, human serum had the highest and sheep serum the lowest capacity for corticosteroid binding.

Studies made on the properties of corticosteroid protein complexes showed that the eluted corticosteroids were unaltered physically; there was an interchange between the bound and added corticosteroid; there was no competition for sites between the individual corticosteroids. It was shown in human sera that, at maximum binding between corticosterone-4-C^{14} and albumin, approximately 5 albumin molecules per 1 corticosterone molecule were involved.

The findings indicate the complexity of the characteristics of the protein carrier mechanism for steroid hormones in diverse animal species.

INTRODUCTION.

The evidence of the binding capacity of corticosteroids to plasma proteins is becoming more important for the understanding of the biological activity of such complexes. A number of workers have demonstrated by different methods that the binding of steroids to protein fractions can take place either *in vivo* (Roberts and Szego, 1946; Peterson and Wyngaarden, 1955; Daughaday, 1956, 1958; Antoniades, Pennell, Slaunwhite and Sandberg, 1957) or *in vitro* (Roberts and Szego, 1946; Szego, 1953; Eik-Nes, Schellman, Lumry and Samuels, 1954; Sandberg, Slaunwhite and Antoniades, 1957). It was shown in recent investigations by Slaunwhite and Sandberg (1959) and Daughaday (1958) that human plasma contains a "special" protein which binds small amounts of cortisol and corticosterone much more strongly than the other plasma protein fractions.

During the course of a separate investigation on the corticosteroid secretion in sheep (McDonald and Reich, 1959) it was noticed that when excess amounts

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[2] Present address: Baker Medical Research Institute, Alfred Hospital, Melbourne, S.1.

of cortisol or corticosterone were added to sheep's plasma the binding capacity, as indicated by movement in electrophoretic field with the different protein fractions, was very slight compared with human plasma. This finding suggested the possibility that the capacity of proteins to associate with corticosteroids varies with the animal type. In this study using the paper electrophoresis method, an investigation was made, (a) to establish the maximum *in vitro* capacity of serum protein fractions for the association of cortisol-4-C^{14} and corticosterone-4-C^{14} in different animal types; (b) to attempt to determine the factors concerned in the association of corticosteroids with serum proteins by studying both the corticosteroid and protein part of the complex. The animal types chosen, hereafter designated as "species", were those most frequently used in experiments for studying adrenal function.

Materials and Methods.

The blood samples were collected from healthy humans and animals. Venous blood was used from man, dogs, sheep and rabbits, and the rats were bled from the heart under ether anaesthesia. The separated sera were analysed preferably the same day, although storage at 3° C. for two weeks did not alter the binding capacity.

Preparation of steroid-serum mixtures.

From 4 to 21 μg. of corticosteroid in ethanol were used in the experiments with human sera, and 4 μg. corticosteroid in the experiments with sera of other species. The ethanolic solution was evaporated to dryness under a stream of nitrogen at 35° C. and to the remaining steroid residues 0·1 ml. of serum was added. The mixture was then incubated for 2 hrs. at 37° C. with frequent shaking. Although binding of corticosteroids with serum proteins occurred at room temperatures, incubation was preferred to ensure total binding.

Paper electrophoresis and chromatography.

Paper electrophoresis was carried out with a modified Flynn and de Mayo apparatus in a manner as described previously (Reich, Coats and McDonald, 1955) using barbituric acid-sodium barbiturate buffer (pH 8·6 and ionic strength 0·05 M). The serum-corticosteroid mixtures were applied onto 4 cm. wide Whatman No. 1 filter paper strips and electrophoresis was carried out at room temperature for 16 hrs. at 0·25 m.amp./cm. width. Six strips were run simultaneously in each experiment, enabling comparison of mobilities under similar conditions. On some occasions from 2 to 6 μg. cortisol-4-C^{14} and corticosterone-4-C^{14} were applied on strips alone and also included in the electrophoretic run. These strips were used for the determination of electrophoretic mobility of the free steroids and also for the calibration of the amount of radioactive counts given by a certain quantity of corticosteroid.

After electrophoresis the strips were dried at temperatures between 35° C. and 38° C. and reserved for the analysis of radioactive areas.

To investigate the nature of the steroid protein complex, the strips were subjected to chromatographic separation, after electrophoresis. For this purpose a Whatman No. 1 filter paper was cut into a shape as illustrated in Fig. 1. The mixture was applied on the line XY. The paper areas a, b, c, d and a^1, b^1, c^1, d^1 were rolled up to cd and c^1d^1 respectively and the strip was fitted into the electrophoretic apparatus. After electrophoresis was carried out the paper was dried at room temperature and the projections beyond cc^1 and dd^1 were cut off. To concentrate the corticosteroid on to spots, the paper was suspended vertically and the part below the line cd was immersed into a solvent mixture of ethyl acetate-methanol

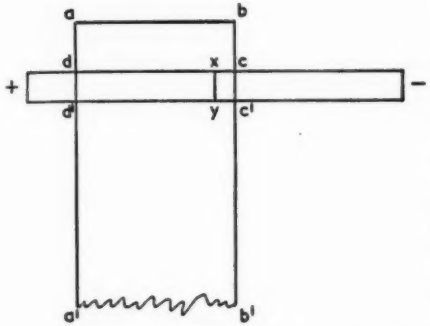

Fig. 1. The shape of the filter paper used in the chromatographic and electrophoretic examinations of the steroid protein complex. See text.

(2:1) which was allowed to move up to line c^1d^1. After drying, the process was repeated at least three times. The paper strip was then subjected to chromatographic separation in toluene 75 p.c. methanol at 30° C. for 2½ hrs. Strips containing pure cortisol-4-C^{14} and corticosterone-4-C^{14} were also included in the run. After drying, all strips were treated with 2N NaOH and heated at a temperature of 80° C. for 2 hrs. The chromatographic procedure and colour reaction of steroids are described in detail elsewhere (Reich, 1958). When viewed in strong ultraviolet light the yellow fluorescing areas on the strip containing the serum corticosteroid mixture revealed mobilities identical with those of the pure steroids.

Radioactivity measurements.

The paper was drawn at a rate of 6 in./hr. between two mica end-window G.M. tubes (20th Century type). A slit width of 0·5 cm. was used, and the combined shielded background of the tubes was 20 counts/min. The radioactivity was measured with an EKCO type ratemeter, using a time constant of 20 sec. The output from the ratemeter was connected to a Record type chart recorder, which also provided the drive for marking the paper, so that the rates of paper strip and chart movements were exactly synchronised.

In quantitative studies done on some sera the strips were analysed for radioactive areas and, using this graph as a reference, the peaks designating the albumin areas were cut out and eluted. The eluates were concentrated and applied on to planchettes, and radioactivity measured using a thin mica end-window G.M. tube and scaler (Reich, 1958).

After radioactive measurements were carried out on the strips they were dyed with bromophenol blue and the positions of the dyed protein fractions were compared with the positions of the radioactive areas as plotted on the chart recorder.

The total protein concentration of the sera was estimated by the copper sulphate specific gravity method (Phillips, van Slyke, Hamilton, Dole, Emerson and Archibald, 1950). The relative concentration of the protein components of the sera was evaluated by means of an "EEL" scanner, and the areas on the graphs were calculated by means of the planimeter.

Ultrafiltration method.

The method was based on that of Lavietes (1937).

Steroids.

Cortisol-4-C^{14} and corticosterone-4-C^{14}, with specific activities of 1·467 millicuries per millimole were obtained from the endocrinology section of the U.S. Public Health Service. The nonradioactive steroids were obtained from Ciba Ltd., Basle, Switzerland. All steroids were checked for chromatographic purity. The standards were prepared in ethyl alcohol.

RESULTS.

The association of cortisol-4-C^{14} and corticosterone-4-C^{14} with protein fractions of the corresponding sera took place in the human, dog, sheep, rabbit and rat. Fig. 2 illustrates the amounts of cortisol-4-C^{14} and corticosterone-4-C^{14} bound to the individual serum protein fractions of different species on addition

of excess amounts of corticosteroid. A marked species difference in the *in vitro* capacity of the serum protein fractions to combine with corticosteroids has been demonstrated. By inspection of the curves human serum had the highest capacity for cortisol-4-C^{14} and corticosterone-4-C^{14} binding and sheep serum the lowest.

In the human sera corticosterone-4-C^{14} associated mainly with albumin, but also with α- and β-globulins. Cortisol-4-C^{14}, on the other hand, associated relatively little with albumin, the radioactivity being mainly detected in the β- and probably the γ-globulin fractions. However, as also described by Daughaday (1956), the radioactivity was not associated only with the particular globulin peaks but seemed to decrease progressively from the point of application, suggesting diffusion effects due to dissociation of the steroid from its moving protein component.

In dog serum the degree of association of corticosteroids was relatively small. Both cortisol-4-C^{14} and corticosterone-4-C^{14} bound mainly with β- and probably γ-globulins and very little with α-globulins and albumin.

In the sheep serum there was little difference in the uptake of cortisol-4-C^{14} and corticosterone-4-C^{14}, there being a slightly greater degree of association with the former. Both corticosteroids bound with albumin and β- and α-globulins, but there was probably no association with the γ-globulin fraction.

In the rabbit serum there was more uptake of corticosterone-4-C^{14} than of cortisol-4-C^{14}, although both corticosteroids appeared to associate with every protein fraction of the serum. Most uptake of corticosterone-4-C^{14} was on the β- and probably γ-globulins and very little on albumin. However, as in human sera, the radioactivity was not confined only to the individual protein components, suggesting diffusion effects.

Two different strains of rats were studied, the mixed (Wistar and albino) and the Sprague Dawley rat. There was minimal binding of corticosteroids with the serum protein fractions of the Sprague Dawley rat. However, in the mixed strain the association of corticosteroids with the serum proteins took place to a relatively large degree when compared with other species (Fig. 2). Binding took place mainly with the β-globulin and albumin when corticosterone-4-C^{14} was added and mainly with α-globulin and albumin when cortisol-4-C^{14} was added.

As indicated in Fig. 2, free cortisol-4-C^{14} or corticosterone-4-C^{14}, when applied on the paper alone, had a slight mobility in the electric field towards the cathode. Due to endo-osmosis, the γ-globulin fraction also had a tendency to move towards the cathode. In the sera of sheep and rats for this reason it was difficult to assess in the graphs which part of the peak was due to the γ-globulin association, and which part was due to the excess corticosteroid. It appeared that association with γ-globulin took place in the human, dog and rabbit, as there was in those mixtures a suggestion of a double peak, and also the curves of radioactivity were much flatter and wider than was the curve

with only free steroid. The association between the γ-globulin and cortisol or corticosterone in human sera was shown in both *in vitro* and *in vivo* experiments by Sandberg *et al.* (1957).

Studies on the properties of corticosteroid protein complexes.

It was first necessary to demonstrate that the corticosteroid when released from its protein component in the different sera was unaltered physically. This was shown by using a method of combined electrophoresis followed by chromatography on the same strip of paper as described. The eluted corticosteroids had the same chromatographic mobility and gave the same colour reactions as did the pure steroids. It was shown also that the protein corticosteroid bond could easily be broken by organic solvents of low polarity, and the corticosteroid could be completely eluted from the protein.

During this study it was noticed that when a concentration of 4 μg. corticosterone-4-C^{14} per 0·1 ml. of human serum was subjected to radioactive measurements after electrophoresis, there was no free steroid left at the origin and most of the radioactivity was on the albumin fraction. To determine the maximum capacity of human serum to bind with corticosteroids quantities of 4, 9·5, 14 and 21 μg. corticosterone-4-C^{14} and quantities of 4, 8, 10 and 12 μg. cortisol-4-C^{14} were added to 0·1 ml. portions of sera from the same individual. The total protein concentration of this serum was 6·67 gm./100 mls. and the albumin to globulin ration was 1·25. It is indicated in Fig. 3 that with corticosterone-4-C^{14} the largest peaks of protein bound radioactivity occurred in sample (c), to which a concentration of 14 μg. per 0·1 ml. serum was added. Although a higher concentration was added to sample (d), there was no further increase in the measured radioactivity of the protein bound peaks. Thus it was concluded that the concentration of approximately 14 μg. per 0·1 ml. of serum was adequate to saturate the binding capacity of this serum. It appeared from the radioactivity measurements that corticosterone, when added to serum at lower concentration (4 μg. per 0·1 ml. serum), was mainly taken up by the albumin with little uptake on the globulins. As more corticosterone-4-C^{14} was added the various globulin fractions also took up more radioactivity. In the case of cortisol-4-C^{14} saturation of all protein fractions took place at a concentration of approximately 10 μg. per 0·1 ml. In contrast to corticosterone, cortisol-4-C^{14}, when added at lower concentrations, bound first with the various globulins, with very little radioactivity on the albumin and, only if higher concentrations were added, the albumin appeared to associate with a larger amount of cortisol-4-C^{14}. It was evident from the distribution of radioactivity on the paper (Fig. 3) that diffusion occurred. This could have resulted from the movement of the smaller protein molecules causing the movement of non-bound steroid in the same direction; and also from the dissociation of weakly bound steroid from the protein component as it moved in the electric field. This would cause the radioactivity baseline to be raised at either end of the paper. For this

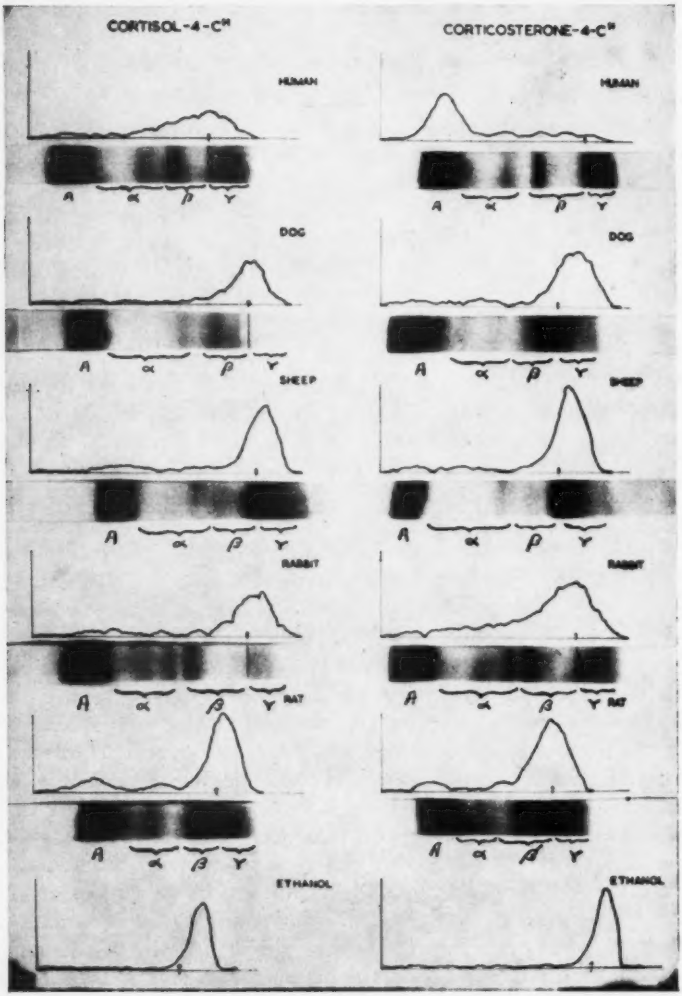

Fig. 2. Effect of addition of excess amounts of cortisol-4-C^{14} and corticosterone-4-C^{14} to the sera of different species. Radioactive measurements of cortisol-4-C^{14} and corticosterone-4-C^{14} after paper electrophoresis in sera of different species and in ethanol. Abscissae: paper strip with stained serum protein fractions. Line on strips and arrow indicate point of application. The areas on strips designate, reading right to left, gamma, beta and alpha globulins and albumin. Ordinates: counting rate.

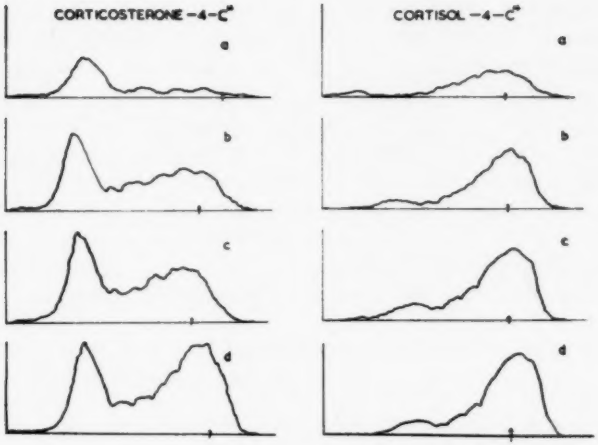

Fig. 3. Effect of addition of increasing amounts of corticosterone-4-C^{14} and cortisol-4-C^{14} to human serum.
Corticosterone-4-C^{14}: (a) 4 µg./0·1 ml.; (b) 9·5 µg./0·1 ml.: (c) 14 µg./0·1 ml.; (d) 21 µg./0·1 ml.
Cortisol-4-C^{14}: (a) 4 µg./0·1 ml.; (b) 8 µg./0·1 ml; (c) 10 µg./0·1 ml.; (d) 12 µg./0·1 ml.
Abscissae: Separated protein fractions, arrow point of application. Furthest fraction from right to left indicating albumin.
Ordinates: Counting Rate.

reason it was possible to calculate the maximum binding capacity only for albumin, as the level of the baseline was very low relative to total counts on the albumin fraction.

To investigate the significance of this diffusion effect, ultrafiltration studies were carried out on two human and two sheep sera, using the anaerobic method of Lavietes (1937). In the human sera the total protein bound corticosterone-4-C^{14} determined by this method approximately equalled that determined by the electrophoretic method. In sheep sera, although the total protein bound corticosterone-4-C^{14} was about 1/10 that of human, it was considerably in excess of that determined by the electrophoretic method. This suggests that the strength of the protein-corticosteroid bond varies between different species, and hence inferences from comparison between the findings of ultrafiltration and electrophoresis may be valid for one species, but not for another.

When sera from different human individuals with various total protein concentrations were used, it was noticed that there was a considerable variation in the quantity of corticosterone-4-C^{14} bound to the individual protein fractions. Thus an attempt was made to determine the concentration of corticosterone-4-C^{14} required for saturation of a certain concentration of albumin. In this

study the radioactive peaks designating the albumin areas were located and cut out from the strip. The corticosterone-4-C^{14} was then eluted from these sections with an ethyl acetate:methanol mixture (2:1) and the eluates were concentrated and transferred to planchettes for radioactivity analysis. The results are illustrated in Table 1. Under the experimental conditions the amount of corticosterone-4-C^{14} required to saturate 1 gm. of human albumin ranged between 1·07 and 1·27 mg. Thus on a molecular basis 4 to 5 molecules of albumin combined with 1 molecule of corticosterone. Daughaday (1956a) found that at low steroid concentration the steroid to protein molecular ratios were also far below unity. It seems likely that the total albumin fraction took part in the association with corticosterone and not only a specific protein fraction moving with the same mobility as albumin, as suggested by Daughaday (1956). Otherwise it would require that the concentration of such a protein would change to exactly the same degree as the concentration of albumin in the individual sera.

TABLE 1.

Quantity of corticosterone-4-C^{14} bound to normal human serum albumin.

Subject	Total serum albumin	Corticosterone-4-C^{14} mg. bound per 1 gm. albumin	Albumin molecules* per one molecule of corticosterone-4-C^{14}
G.E.	4·58	1·15	5
J.B.	3·80	1·20	5
V.T.	3·23	1·07	5
M.E.	3·25	1·21	5
B.C.	2·67	1·27	4
D.S.	3·10	1·21	5
P.G.	2·60	1·11	5
E.R.	3·00	1·25	4

* Molecular weight of albumin, 67,000.
Molecular weight of corticosterone 364·4.

To get an indication whether an exchange could occur between the bound and added corticosteroid, the following experiments were carried out. Human sera were saturated with non-labelled corticosterone or non-labelled cortisol. The sera were incubated for 2 hrs. at 37° C. and the same saturation quantity of corticosterone-4-C^{14} or cortisol-4-C^{14} was added, followed by further incubation for 2 hrs. When the sera were analysed it was noticed that every protein fraction contained radioactive corticosteroid, but the amount of radioactivity was only 40-50 p.c. of the total radioactivity as compared with sera which contained only radioactive corticosteroids. This indicates that after 2 hrs. at the saturation concentration there is approximately equal interchange between the bound and unbound corticosteroid molecules.

Investigating further the nature of the corticosteroid protein complex, experiments were carried out to determine the extent to which one corticosteroid

might displace another corticosteroid from the protein. Human serum was saturated with non-labelled cortisol followed by the addition of corticosterone-4-C^{14}. Similarly, sera were saturated first with non-labelled corticosterone followed by the addition of cortisol-4-C^{14}. To another serum approximately 15 μg. aldosterone were added followed by 14 μg. corticosterone-4-C^{14}. Radioactivity measurements carried out after electrophoresis revealed in every case both qualitatively and quantitatively similar patterns to those found when only the radioactive cortisol-4-C^{14} and corticosterone-4-C^{14} were added. These experiments indicate that at high concentration there are specific sites on the protein molecule to bind with a particular type of corticosteroid and that there is no competition for these sites between the individual corticosteroids.

DISCUSSION.

The experiments indicate that association of cortisol-4-C^{14} with serum protein fractions, as shown by paper electrophoresis, was both quantitatively and qualitatively characteristic to the species. Although rabbits and rats mainly secrete corticosterone (Bush, 1953), cortisol was also found to associate with the serum proteins of these species. In the human and the sheep there was some variation in the amounts of corticosteroids bound to serum proteins of the individual samples. On the other hand, in rabbits and rats the amounts of bound corticosteroid were remarkably uniform between individuals. The fluctuations in the amounts of corticosteroids bound to protein components could either be due to the weak interaction of such a complex, or else to differences in the amounts of the protein fractions in the individual sera. However, there was no significant variation in the amount of total protein in the sera of different species, the concentrations ranging from 5·5 to 7·5 gm./100 ml. which was within the range of individual variation for a particular species. Although the concentrations of total albumin were relatively not very variable in the species studied, human albumin had by far the greatest capacity for corticosterone binding. It was interesting that the maximum amount of albumin bound corticosterone was fairly constant in sera of different human individuals, being approximately 1 molecule corticosterone per 4 to 5 molecules of albumin.

Most workers by using the methods indicated—Daughaday (1956, equilibrium paper electrophoresis; and 1956a, dialysis); Sandberg, Slaunwhite and Antoniades (1957, ultrafiltration and dialysis); Antoniades, Pennell, Slaunwhite and Sandberg (1957, ultrafiltration and dialysis)—found that cortisol-4-C^{14} associated mainly with human albumin. By using paper electrophoretic separation Westphal, Firschein and Pearce (1956) and Upton and Bondy (1958) showed that almost the total cortisol-4-C^{14} added to the serum stayed at the origin. Daughaday (1956) has shown by equilibrium paper electrophoresis that the affinity of corticosterone for albumin is more than twice that of hydrocortisone. In studies done on rat serum, Szego (1957) showed that there was a significant

increase in the steroid albumin binding if the mixture was incubated with surviving rat liver, and stressed the rôle of a liver enzyme system in the formation of the protein corticosteroid complex. No mention was made by this worker concerning the physical properties of the corticosteroids after being released from the protein complex. In the present investigation, in every species the bound corticosteroid could be eluted from its protein component without being physically altered by the use of organic solvents of little polarity. Thus this bond was of a very loose nature. It was reported by Schellman, Lumry and Samuels (1954) and Westphal (1955) that the forces between the steroid albumin complex are weak adsorptive forces of a magnitude about 5 kilocalories. Upton and Bondy (1958) found in their studies that cortisol is bound to the protein with a tight bond at low steroid concentration and a loose bond at high steroid concentration. In the present study the concentrations used would fall into their loose bond category. Westphal *et al.* (1956) investigated the possibility of the detachment of cortisol from its albumin component due to adsorption to filter paper, by comparing the results of electrophoresis experiments with paper chromatography and found that the different results obtained by the two methods were due to the very weak bond and not the adsorptive power of the filter paper. It appeared, when the electrophoretic method was compared with the ultrafiltration method, that the magnitude of these forces could vary in different species.

It is well known that the protein fractions of the sera of different species vary both in the electrophoretic mobility and the number of fractions separated and, therefore, it appeared that the protein was the determining factor in the formation of the corticosteroid protein complex. Riegel and Mueller (1954) have shown that this bond is determined by the number of ionized groups outside the protein molecule. No competition for binding sites on protein was noted between cortisol, corticosterone, or aldosterone, and it was therefore concluded that at high steroid concentrations there are specific sites on the protein molecule for the binding of the individual corticosteroids.

The explanation of the results on the basis of *in vivo* systems would be purely speculative, mainly because the concentrations of corticosteroids used in the experiments were well above physiological levels. However, the patterns in Fig. 2 indicate that, by the method employed, the serum proteins of every species were capable of binding a quantity of corticosteroid which greatly exceeds the maximal peripheral concentration. Daughaday (1956b) and Antoniades *et al.* (1957) found that the corticosteroids introduced into the human vascular system bound quickly with plasma proteins. When amounts similar to the physiological concentrations were injected into human subjects Daughaday found that 94 p.c. of the corticosteroid was protein bound. Antoniades *et al.* injected into humans amounts of 0·5 to 0·7 mg. of corticosterone or cortisol, and although these caused plasma levels in excess of normal there was essentially complete binding to plasma proteins.

Whether the maximum *in vivo* capacity of the serum proteins of a certain species to associate with corticosteroids is the same as the *in vitro* one is difficult to predict. Upton and Bondy (1958) state there is no qualitative difference between the binding of exogenous and endogenous steroid to human plasma. Daughaday (1956a) found that the degree of binding of cortisol was of the same order of magnitude when given to normal humans by mouth or when trace amounts of cortisol-4-C^{14} were added to plasma. This author (1956b) also stated that the binding of cortisol is purely a physical process not requiring cellular activity.

Daughaday (1958) and Mills and Bartter (1959) found by the method of ultrafiltration that human plasma contains a specific binding globulin which has a high affinity for cortisol at low concentrations. Slaunwhite and Sandberg (1959) isolated a specific protein which has a very high affinity for cortisol, but it can bind also other corticosteroids to a lesser extent. This protein, which they named transcortin, had electrophoretic properties of α-globulin and it was found also in varying degrees in dogs, rabbits, guinea-pigs and rats. These authors suggest that the transcortin-bound cortisol is inactive, and only the non-transcortin-bound cortisol possesses physiological activity. If all the corticosteroids in the vascular system were protein bound, it would be interesting to study what is the ratio of transcortin-bound corticosteroid to weakly bound in different species, as it appears that the capacity of weak binding is very variable from one species to another.

Resulting from the experimental findings presented, it was interesting to note the difference in the removal rate of corticosteroids from the circulation of different species. It was found by Brown, Willardson, Samuels and Tyler (1954); Peterson, Wyngaarden, Guerra, Brodie and Bunim (1955), Ayres, Garrod, Tait, Tait and Walker (1957), and Nugent, Eik-Nes and Tyler (1959) that the half life of cortisol in humans is approximately 90-113 min., in dogs 52 min. (Collins, Forist and Nadolski, 1956), in sheep 20-30 min. (Ferguson, personal communication), and in rats up to 32 min. (Ulrich and Long, 1956). If one compares the capacity of the protein fractions of the sera of these species to associate with cortisol-4-C^{14}, then human serum associates with the largest amount and sheep serum with the smallest amount of cortisol. In their studies, Mills and Bartter (1959) noticed that following oestrogen administration there was up to 80 p.c. increase in the binding of cortisol by the specific plasma proteins. Concurrently, the half life of cortisol increased from 75 to 114 min. Hence, there is a possibility that the increased ability of the peripheral plasma to combine with corticosteroids in different species may have some relationship to the longer half life of the steroid, being so protected from destruction by the liver.

These findings illustrate the complexity of the characteristics of the protein carrier mechanism for corticosteroid hormones in diverse animal species and

indicate the necessity for specification of the individual species in the study of corticosteroid function.

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